Universidade de Aveiro Departamento de Química
2008

Joana Patrícia do Vale Afonso

VALORIZAÇÃO DE EXTRACTOS DE GRAINHA DE UVA: ÓLEO E PROCIANIDINAS

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dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Engenharia Química, realizada sob a orientação científica do Doutor Carlos Silva, Professor auxiliar do Departamento de Química da Universidade de Aveiro e co-orientação científica do Doutor Manuel António Coimbra, Professor Associado com Agregação Do Departamento de Química da Universidade de Aveiro.

Ao meu Avô

o júri

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palavras-chave

Enzima Conversora da Angiotensina I, Inibidores da Enzima Conversora da Angiotensina I, Procianidinas, Grainha de Uva, Óleo, Extracção, Caracterização.

resumo

A valorização de produtos secundários na indústria, tem sido alvo de um crescente interesse, devido a razões ambientais e económicas. A grainha de uva é um produto secundário da vitivinicultura, pelo que existe uma crescente necessidade de encontrar aplicação e criar valor para algo que é normalmente visto como um resíduo.

A grainha da uva possui dois componentes importantes: Óleo e Compostos Polifenólicos. Estes dois componentes foram alvo de estudo neste trabalho. O óleo e os compostos polifenólicos, da grainha da uva da variedade branca Chardonnay, *Vitis vinífera*, L., foram extraídos e caracterizados.

O óleo da grainha da uva (11,6% de óleo no conteúdo total da grainha de uva) apresentou 77,2% de ácidos polinsaturados na forma de triglicerideos, características que tornam este óleo num produto com alto valor nutricional.

Os compostos polifenólicos foram extraídos com dois solventes, metanol e a mistura de acetona/água, tendo sido obtidos 25.5% de extracto rico em compostos polifenólicos.

O extracto rico em compostos polifenólicos foi fraccionado em relação ao grau médio de polimerização e caracterizado. Os compostos polifenólicos presentes na grainha da uva são flavan-3-óis, na sua maioria procianidinas, polímeros de unidades de (+)-catequina, (-)-epicatequina e (-)-epicatequina-3-*O*-gallato.

Quatro das extracções fraccionadas foram testadas como possíveis inibidores da enzima conversora da angiotensina I (ECA), tendo-se observado uma inibição de quase 100% para 30 mg/mL de extracto. Os resultados sugerem uma relação entre o grau de polimerização das procianidinas nos extractos e a inibição da ECA, assim como um possível efeito sinergístico dos compostos presentes no extracto. Os compostos polifenólicos possuem assim um possível papel na prevenção de doenças cardiovasculares.

keywords

Angiotensin I-Converting Enzyme; ACE inhibitor; Procyanidins; Grape Seed; Hypertension; Oil, Extraction, Characterization

abstract

Valorization of by-products in industry is an increasing concern for Environmental and Economical reasons. Grape seeds are a by-product of wine industry and a there's a need to find application and value to what is normally seen as a waste. Grape seeds have two important components Oil and Phenolic Content. Both components were studied in this work. Extraction and characterization was carried for both components.

Grape seed oil (11.6 % of oil in total grape seed content) presented 77.2% of polyunsaturated acids, in the form of triglycerides, which give this oil, characteristics of edible and quality oil.

Polyphenolic compounds were extracted from grape seed *Vitis vinifera* L, white variety Chardonnay, with two solvents, methanol and a mixture of acetone/water. A 25.5 % of a rich-extract in polyphenolic compounds was obtained. The polyphenolic rich-extract was fractionated in terms of the average degree of polymerization and characterized. Phenolic compounds present in grape seeds were flavan-3-ol, mainly procyanidins, in polimerized forms of (+)-catechin, (-)-epicatechin and (-)-epicatechin-3-*O*-gallate units.

Four of the fractions obtained were tested as inhibitors of Angiotensin I-Converting Enzyme and a strong inhibition was observed, almost 100 % of inhibiton for 30 mg/mL of extract. A connection between the average degree of polymerization of polyphenolic extracts and their strength to inhibit ACE seems to exist, as well as a possible synergistic effect of compounds present in the extract. Polyphenolic compounds of grape seed assume a possible role in the prevention of cardiovascular diseases.

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Nomenclature

ACE	Angiotensin I Converting Enzyme
Ang II	Angiotensin II
A-PCE	Procyanidins crude extract obtained with acetone/water
Ang	Angiotensin I
BK	Bradykinin
$\overline{d_p}$	Average particle diameter
DPn	Average degree of polymerization
FTIR	Fourier transform infrared spectroscopy
FID	Flame Ionization Detection
GS_{DM}	Grape Seed Dried Mass
gACE	Angiotensin I-Converting Enzyme expressed in somatic tissue
GC-FID	Gas Chromatography with Flame Ionization
GC-MS	Gas Chromatography with Mass Spectrophotometry
GC	Gas Chromatography
Hip-His-Leu	N-hippuryl-L-histidyl-L-leucine
His-Leu	Histidyl-leucine
HPLC	High performance liquid chromatography
$I(\%)$	ACE inhibition
IC_{50}	Inhibitor concentration for which $I = 50\%$;
K_m	Michaelis Menten constant
LDL	Low-density lipoprotein
M- PCE	Procyanidins crude extract obtained with methanol
O_{DM}	Oil Dried Mass
PC_{DM}	Phenolic Content Dried Mass
PCA	Perchloric acid
PCE	Procyanidins crude extract
RAS	Renin Angiotensin System
EPA	United States Environmental Protection Agency
SFE	Supercritical fluid extraction
sACE	Angiotensin I-Converting Enzyme expressed in germinal cells

SEM	standard error mean
S	Substrate
$v\max$	Maximal initial velocity
v_0	Initial velocity
WA-Fx.x	Procyanidins fraction x.x extracted from white grape seeds with acetone/water
WM-Fx.x,	Procyanidins fraction x.x extracted from white grape seeds with methanol

Chapter 1 – Introduction

In recent years, a lot of interest has developed around the research on the industrial application of feedstock from renewable resources, because sustainability will become increasingly important for the chemical industry. Such fact lead to the novel concept of «Biorefinery», in which bioresources such as agriculture or forest biomass are processed to produce energy and a wide variety of precursor chemicals and bio-based materials, similar to the modern petroleum refineries[1,2].

Usually a crop, as it is, is not applicable for industrial use. Industry is only interested in a certain part, or in a chemical or physical component of the crop, which therefore has to be processed in the biorefinery. The different fractions thus produced are then available and sold to various industries, or partially recycled to agriculture, as cattle feed or as organic fertilizer.

In this work we focus grape seed, which is an important by-product of the winemaking production, reaching approximately 15% (w/w) of the musts from wine fermentation. This topic is particularly interesting for Portugal where the production and trade of wine products play an important role in the economy. For instance, between 1999 and 2002, Portuguese production was of 7 232 000 hL [3], which gave rise to nearly 72 000 ton of grape seed must.

Grape seeds are a natural source of oil and polyphenolic compounds (mainly, procyanidins), with nutritional characteristics and health benefits, such as antioxidant properties, low risk of heart failure, diabetes and hypertension. They contain around 15% (w/w) of oil, with high level of unsaturated fatty acids [4], and 7% of polyphenolic compounds (mainly procyanidins)[5]. Though grape seed oil has been widely studied, the procyanidins fraction is receiving recent attention.

Both oil and procyanidins extracts of grape seed were analysed in this work. Although the valorisation of the procyanidins fraction was the target of the thesis, the oil has received considerable attention too. Since the phenolic compounds are obtained from defatted seed, the oil had to be extracted first. Hence, we decided to characterize it additionally, in an attempt to perform a complete study of these constituents of grape seed.

With respect to procyanidins, it is known they interact significantly with biologic systems like those of the enzyme-inhibition type. Procyanidins-rich foods have been shown to inhibit the angiotensin I-converting enzyme [6], which suggests the same should occur with procyanidins from grape seeds.

The angiotensin I-converting enzyme (ACE) is a glycoprotein which hydrolyses histidyl-leucine from angiotensin I to the potent vasoconstrictor angiotensin II, and simultaneously inactivates the vasodilator peptide bradykinin [7]. Inhibition of ACE is a therapeutic approach in the treatment of high blood pressure. In this way, an evaluation of the benefit of grape seed procyanidins in cardiovascular diseases was performed as well.

Accordingly, this work comprehended two main lab exercises: (i) the grape seed extraction and characterization of both oil and procyanidins fractions; (ii) evaluation of the inhibitory effect of ACE by this last fraction. In terms of organization, the dissertation is divided into four chapters: Chapter 2 presents the theoretical background and fundamentals on grape seed components, extraction methods, and angiotensin I-converting enzyme action; Chapter 3 is the experimental section, where methodologies and procedures are described; Chapter 4 presents the results obtained and their discussion; finally, most important conclusions are given in Chapter 5.

Chapter 2 – Theoretical Background and Fundamentals

Chapter 2 provides the reader with the fundamental knowledge and theoretical background of this work. This theoretical background and fundamentals chapter is divided in two main sections:

i) Grape seed composition, characteristics and applications, as well the methods of extraction and treatment of two grape seed components: oil and polyphenolic content;

ii) Fundamental notions of angiotensin I-converting enzyme - since polyphenolic extract, from grape seed, was used to evaluate the activity of this enzyme.

2.1. Grape Seeds – Oil and Polyphenolic content

Grape seeds are one of the most valuable by-products of winemaking industry. Grape seeds contain 15% of oil, 35 % fiber, 29 % extractable components including polyphenolic compounds, 11 % proteins, 3% minerals and 7% of water [8], as showed in Figure 1. Mainly, by its contents of oil and polyphenolic compounds, this waste of wine production is an important material in nutrition and chemical industry as well as a putative health benefit.

Oil and polyphenolic compounds are, therefore, relevant components of grape seeds and will be the object of this study.

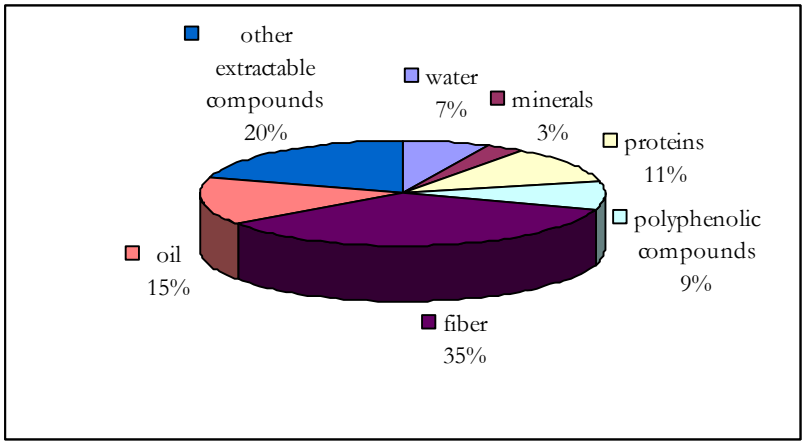


Figure 1 – Grape seed composition

2.1.1. Grape seed oil

Oils and fats are considered important renewable raw material in chemical industry. World oil production between 1996 and 2000 had reached the average annual amount of $105.0 \times 10^6 ton$ and it is estimated a production increasing in the years 2016 to 2020 to $184.7 \times 10^6 ton$ [9]. Of the total oil production, 80% are from vegetable origin, such as soybean, palm oil, rapeseed and sunflower. Approximately 80% of oil is used as human food; 6% as feed and the remaining 14% are used by industry [9].

The production of oil from grape seeds can result in interesting edible oils with a comparable health benefit as other vegetable oils. Grape seed oil is becoming increasingly popular for culinary, pharmaceutical, cosmetics, and medical purposes, primarily due to the high level of unsaturated fatty

acids, namely oleic and linoleic acids [10]. Such topics are particularly interesting for countries where the production and trade of wine products play an important role in the economy.

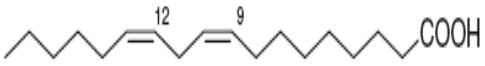
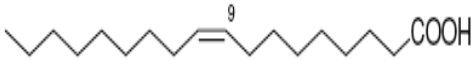
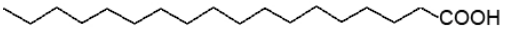
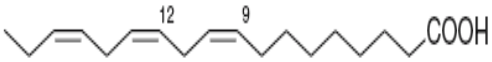
2.1.1.1. Grape Seed Oil Composition

Grape seed contain 7 to 20 % of oil [11] [8,12], mainly of polyunsaturated fatty acids. In fact, fatty acids represent 90% of grape seed oil total composition. Grape seed oil also contains unsaponifiables rich in phenols like tocopherols and steroids (0.8 to 1.5%)[13]. Grape seed commercial oil shows 80 % of polyunsaturated fatty acids, where 57 % is of linoleic acid, 22 % is of oleic acid and 0.4% of linolenic acid.

Baydar et al. [12] studied the composition of oils of different varieties of grape seeds (*Vitis vinifera* L), finding that in the variety of grapes seeds studied the linoleic acid was the fatty acid with major percentage (63–69%), followed by the oleic acid with 21–16%. The other fatty acids present in grape seeds were palmitic acid (9–10%), stearic acid (4–5%) and traces of linolenic acid (0.26–0.35%). Unsaturated fatty acids (oleic acid, linoleic acid and linolenic acid) correspond to 85% of the total fatty acids present in the oil from grape seeds. Some polyunsaturated fatty acids, such as linoleic and linolenic acids, are essential to human body since they can not be synthesized[12], which suggest that the inclusion of grape seed oil in the human diet could bring health benefits. Unsaturated fatty acids are very important for the stability of oils because of chemical reactions in double bonds. The rate of oxidation reactions depends on the number of double bonds in the carbon chain [12]. Low levels of linolenic acid could at first seem a disadvantage (since it is not synthesized endogenously), however lower levels of linolenic acids are desirable in nutritional oils since huge quantities of this fatty acid could produce undesirable odours and taste in the oil. Moreover, linolenic acid is easily oxidized due to the presence of three double bonds in its hidrocarbon chain, and would have a short stability in a linolenic rich-oil [12].

Table 1 presents the structure of fatty acids present in grape seed oils. Percentage of fatty acids found in grape seeds by Baydar et al. [12], is also presented.

Table 1 – Chemical structure of fatty acids present in grape seeds. Percentage of fatty acids in *Vitis vinifera* L varieties, studied by Baydar et al. [12].

Fatty Acids	Type	Chemical Structure	(%)
Linoleic acid (C18:2)	$\omega 6$ -unsaturated		63.3 – 69.7
Oleic Acid (C18:1)	$\omega 9$ -unsaturated		16.1 – 21.6
Palmitic Acid (C16:0)	saturated		8.8 – 10.2
Stearic Acid (C18:0)	saturated		4.0 – 4.7
Linolenic Acid (C18:3)	$\omega 3$ -unsaturated		0.26 – 0.35

2.1.1.2. Extraction of Grape Seed Oil

Grape seed oil has been traditionally recovered by hydraulic pressing and solvent extraction, mainly with *n*-hexane. The excellent solubility of the oil in *n*-hexane, the fairly narrow boiling point range, 63-69ºC, and the easy recovery makes *n*-hexane a preferential solvent [14]. However, *n*-hexane is listed as nº1 in the list of 189 hazardous air pollutants by US Environmental Protection Agency [15]. The concerns with health, environment and safety led to an increase in the use of alternative solvents such as isopropanol and ethanol. Nevertheless, the use of alternative solvents often results in a decrease of the solute from the solvent due to the low affinity between solute and solvent. The costs associated to the use of alternative solvents can also be higher since, to increase the polarity of liquid phase is frequently added a co-solvent [16]. According to Mamidipally and Liu [15], using (+)-limonene and *n*-hexane in the extraction of oil from rice bran, a significant amount of oil is obtained. This mixture extracts more, than the *n*-hexane alone, under any given set of conditions.

- **Conventional Soxhlet extraction**

Classic extraction techniques of compounds from material matrixes are based on the appropriate choice of the solvent coupled with the use of heat and/or agitation. The extraction with soxhlet was, during a long time, the main extraction method and the main reference for evaluating the performance of other liquid-solid extractions [14].

Figure 2 represents a soxhlet extraction system. Soxhlet extraction system consists in a solvent flask connected to a thimble-holder – the soxhlet extractor – with the material to extract, and a condenser responsible for solvent recovery.

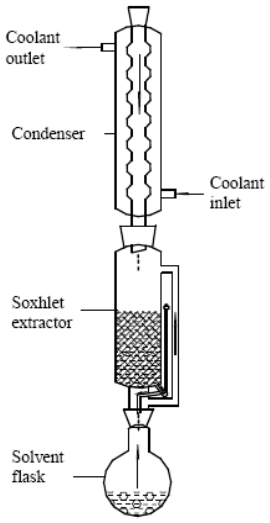


Figure 2 – Conventional soxhlet extraction apparatus

The heating in the solvent flask leads to evaporation of fresh solvent which contacts with the material to extract in the thimble-holder. When the liquid reaches the overflow level, a siphon removes the solution from the thimble-holder and unloads it back into the solvent flask. The extracted solute is carried into the bulk liquid. Solute is left in the flask and the fresh solvent passes back into the solid bed. The operation is repeated until complete extraction is achieved [14].

In soxhlet extraction an important factor is the choice of the solvent to use. Hexane is the most commonly solvent use to extract oil from biomaterials. A low range in boiling point (63-69ºC), a good solubility of the oil in this solvent and easily recovery makes hexane an excellence choice. However, health, safety and environmental concerns, as former appointed, leads to the use of alternative solvents. Still, the use of alternative solvent, may lead to decrease in the solute recuperation due to low affinity between solute and solvent. Alternative solvent costs could become higher, since to increase liquid phase polarity, a co-solvent is usually added. The choice of the solvent has to have into account all these factors.

The matrix characteristics and particle size are also important factors since internal diffusion is the extraction limiting step. Luque-Garcia and Luque de Castro[17], refer that in the extraction of oleaginous seeds with 0.4 mm particles, 2 hours were needed for extracting 99%, while for 2.0 mm particles for the same yield a 12 hours extraction is needed. Milling the seeds greatly improves efficiency, since this operation breakdowns the vegetable cells and augments interfacial area improving mass transfer [18].The yield of the process may be significantly increased by mechanical or thermal conditioning.

In soxhlet extraction, the extraction temperature and evaporation are factors that influence final product quality. The boiling points can be decreased with vacuum in solvent recuperation. Thus, soxhlet extraction presents some advantages: i) change of transfer equilibrium (allows a repeated fresh solvent contact with solid matrix); ii) maintenance of a relative high extraction temperature; iii) absence of last filtration step; iv) simplicity and low cost associated; and some disadvantages: i) long extraction times are required; ii) large solvent quantities; iii) absence of agitation in the soxhlet (which would allow a better contact between solvent and solid matrix, increasing the extraction process); iv) the huge quantity of solvent requires a last step of product evaporation/concentration; v) possible thermal decomposition of the target compounds – as the extraction occurs in boiling point range for a long period of time [14].

Extraction methodologies have been developed to improve operation conditions of the extraction. Reduce extraction time, decrease solvents consume and increase quality of the extracts is an objective of new methodologies [14]. Along the conventional method with soxhlet are emerging new methodologies as sonication, microwave and supercritical fluid extraction.

2.1.1.3. Characterization of Grape Seed Oil

After extraction, oil characterization permits quality control and potential application based on the oil characteristics. Different composition leads to different applications in industry, health and nutrition. While olive oil is almost exclusive used in nutrition, soybean and corn oil are also used as biodiesel.

Characterization of grape seed oil normally is carried with the Gas Chromatography–Flame Ionization Detection (GC-FID) analysis after transesterification of triglycerides present in oil. In fact, the fatty acids present in grape seed oil are mostly in the form of triglycerides. A derivatization of the the oil is required prior the injection on the GC. The transesterification is the process of exchanging the alcohol group of an ester compound with another alcohol. These reactions are often catalyzed by

the addition of an acid or base. Acids can catalyze the reaction by donating a proton to the carbonyl group, thus making it more reactive, while bases can catalyze the reaction by removing a proton from the alcohol, and also making it more reactive.

The transesterification of oils is showed in Figure 3. A triglyceride reacts with an alcohol in the presence of a strong acid or base, producing a mixture of fatty acids, alkyl esters, and glycerol [19].

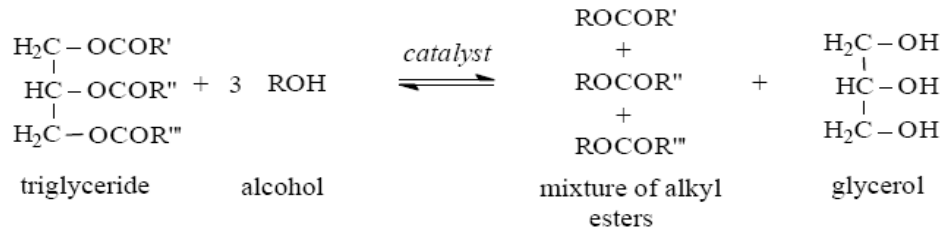


Figure 3 – Transesterification of oils

The overall process is a sequence of three consecutive and reversible reactions, in which di- and monoglycerols are formed as intermediates. Several aspects including the type of catalyst (alkali or acid), alcohol/oil ratio, temperature, purity of the reactants (mainly water content) and free fatty acid content have influence on the course of the transesterification. After transesterification, the mixture of alkyl esters is easily identified by gas chromatography separation and FID detection.

2.1.2. Grape Seed PolyPhenolic Compounds

Polyphenolic compounds are a class of phenolic products present in nature and available in a wide variety of fruits, vegetables, seeds, flowers and barks. In the last years these compounds had been amply studied for their antioxidative, anti-inflammatory, and anticarcinogenic activity. Epidemiological and experimental studies in animals and humans suggest that flavonoids may reduce risk of cardiovascular diseases [20] [21] [22], and cancers [23]. A balanced diet abundant in foods of plant sources can significantly slow down the development of cardiovascular diseases [24].

An aromatic ring linked to a hydroxyl group (-OH) constitute the phenolic group. Polyphenolic present more than one hydroxyl group in one aromatic ring. Polyphenolics are plant secondary metabolic products and comprise one of the largest and most ubiquitous groups known in vegetable kingdom with more than 4000 structures already identified [24].

One important group of polyphenols is flavonoids. Flavonoids follow a basic structure of C6-C3-C6, being two aromatic rings (A and B) connected by one heterocyclic with and oxygen atom.

Flavonoids are divided in subclasses based on the rings B and C connections as well saturation degree, oxidation and hydroxylation of ring C.

Flavanols is a subgroup of flavonoids. Flavan-3-ols are flavanols with a hydroxyl group connected to carbon 3 (Figure 4). These flavanols have a particular interest since grape seeds are a rich source of flavan-3-ols.

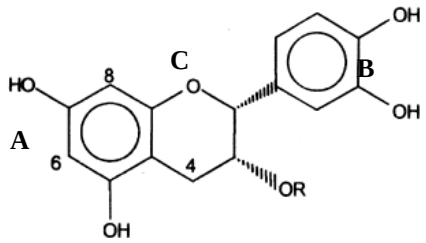


Figure 4 – Flavanol structure

2.1.2.1. Grape Seed Phenolic Composition

Polyphenolic compounds concentration in grape seeds depend on the variety of grapevine and is influenced by viticultural and environmental factors. Montealegre et al, [25], studied the composition of ten grape, *Vitis vinifera* L, varieties grown in a warm climate. The seeds components comprised almost exclusively flavan-3-ols with concentration ranges of 330-1390mg/kg grape. Fuleki and Ricardo da Silva [26], studied the phenolic composition from grape cultivars from Ontario and reported the same cultivar influence in flavan-3-ol composition. The percentage range of flavan-3-ols and procyanidins in seeds was 0.80-17.68 %.

Grape seeds are, thus, composed by monomers, (+)-catechin, (-)-epicatechin, and (-)-epicatechin-3-*O*-gallate, dimers and trimers procyanidins and substantial quantities of highly polymerized procyanidins. Prieur et al [27] found that 55 % of the procyanidins in grape seeds consisted of more than five monomers units. The structures of grape dimeric and trimeric procyanidins mostly consist in (+)-catechin and (-)-epicatechin units, linked by C4-C8 or C4-C6 bonds and sometimes esterified by gallic acid on the epicatechin moieties, the (-)-epicatechin-3-*O*-gallate, as shown in Figure 5.

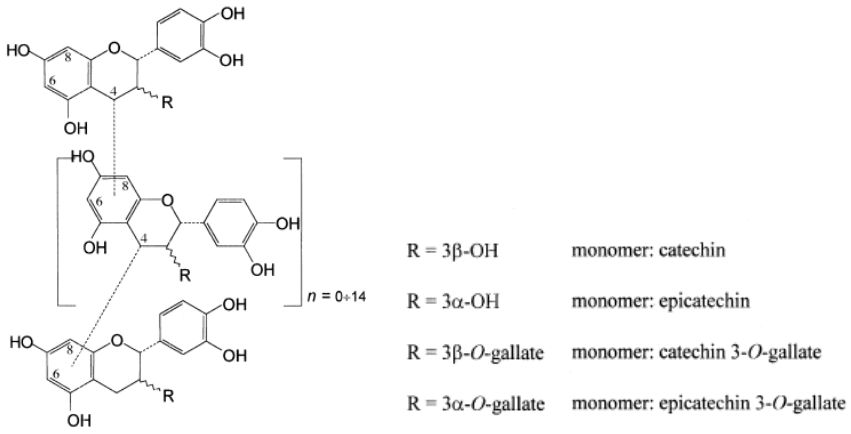


Figure 5 – Procyanidin structure

2.1.2.2. Extraction of Grape Seed Polyhenolic Compounds

Polyphenolic compounds are usually extracted with solvents as water, methanol, ethanol and acetone or a mixture of these solvents. Molecules containing glycosidic residues are soluble in water. Aglycons (non-sugar moiety) show low polarity and are therefore more soluble in other solvents. The specificity of each compound and the increase of the extraction yield can be achieved with a combination of several solvents [28].The extraction of polyphenols is dependant upon two actions, the dissolution of each polyphenolic compound at cellular level in the plant material matrix, and their diffusion in the external solvent medium.

An extraction divided by steps is normally used, exploring a change of solvents in each extraction step. Guyot et al. refers a first extraction with a non polar solvent (hexane), to extract oils, fats, and steroids, followed by subsequent methanol extraction of organic acids and polyphenolic compounds of low molecular weight. Finally an extraction with a mixture of acetone-water is used to extract polymeric procyanidins.

Pekic et al. [29] had demonstrated that ethyl acetate can be used to extract polyphenols, namely catechins and procyanidins from grape seeds. The mixture of this solvent with water (co-solvent) increases significatively the extraction yield, due to the increase of permeability in the grape seed. The seed saturated with water allows a higher penetration of the solvent increasing consequently the extraction yield. After saturation (10% of water) an increase in the % of water does not increase significatively the extraction.

However, extraction methods using organic solvents have a major disadvantage for the putative role of these compounds in human nutrition. Organic solvents are in majority toxic compounds and for that have a restrict use.

As for oil extraction, supercritical extraction appears as an alternative to organic solvents methods. This technique uses as solvent carbon dioxide in the supercritical condition, an innocuous gas to human being which makes its use advisable for human nutrition [30]. Supercritical extraction combines the advantage of gas diffusion with the high power of solvatation of a liquid. Any oxidative effect that can occur during extraction is minimized. However, carbon dioxide is a highly non polar gas and polyphenolic molecules are polar. This problem is solved with an increase in the solvent density, therefore carbon dioxide is used at high pressions and in the presence of a co-solvent to support extraction [28]. The influence of a co-solvent in supercritical extraction of xanthines was reported by Li and Hartland [30]. This study indicates that changing the carbon dioxide density, varying pressure and temperature, a limited increase on carbon dioxide solubility effect is achieved. Co-solvent addition can increase the selectivity capacity and increase supercritical extraction applications. This study refers ethanol as a useful co-solvent in supercritical extraction, a mixture of CO_2/ethanol in supercritical conditions form, miscible in any proportion. The final extract can be used in food without extract/co-solvent separation concern. The solubility of catechins in supercritical carbon dioxide increases with the increase of ethanol percentage added to the system.

2.1.2.3. Characterization of Grape Seed Phenolic Compounds

As was said before, flavanols from grape seeds are mostly procyanidins, consisting of the flavan-3-ol units (-)-epicatechin and/or (+)-catechin linked by C4-C8 or C4-C6 bonds (type-B structures), or doubly linked by a C2-C7 ether (acetal) bond (type-A structures). Both monomers can appear galloylated or not.

To characterize the extracts of polyphenolic compounds of grape seeds is important to determine the average degree of polymerization (DPn) of procyanidins. Procyanidins often behave according to their molecular weight and the nature of their constitutive flavanol unit. The average degree of polymerization influences the bitterness and astringency of wine. DPn and the nature of the constitutive units are important structural features that are related to the ability of procyanidins to associate with proteins and polysaccharides [31].

Several methods have been proposed to fractionate oligomeric and polymeric procyanidins. Gel chromatography is a usual method and different gels that can be used. Unfortunately, only oligomers up to five are easily separated with these methods, and irreversible adsorption often occurs which limits the life of the expensive gels. As alternative, a dissolution procedure uses a binary mixture of chloroform and methanol in order to achieve successive precipitations. The different percentages of the binary mixture permits achieve up to 6 fractions of the phenolic extract. An increase on the % of chloroform in the binary mixture leads to a decreasing DPn of the polyphenolic extract fractions.

After procyanidins fractionation according to their degree of polymerization, each fraction needs to be characterized. Several methods have been proposed to determine DPn, but the chemical method, thiolysis degradation is widely use. Thiolysis, consist in the acid-catalyzed cleavage of the interflavanyl linkages of procyanidins in the presence of nucleophile reagent such as toluene-α-thiol [32]. Thiolysis allows distinction between extension and terminal units of procyanidins and when coupled to reverse-phase HPLC (High Perfomance Low Chromatography), permits to calculate the average degree of polymerization and to determine the proportions of the constitutive units in procyanidins fractions. After thiolyse of procyanidins, terminal units are units of flavanols, while extension units are a chain of flavanols units with a thiol group, as shown in Figure 6.

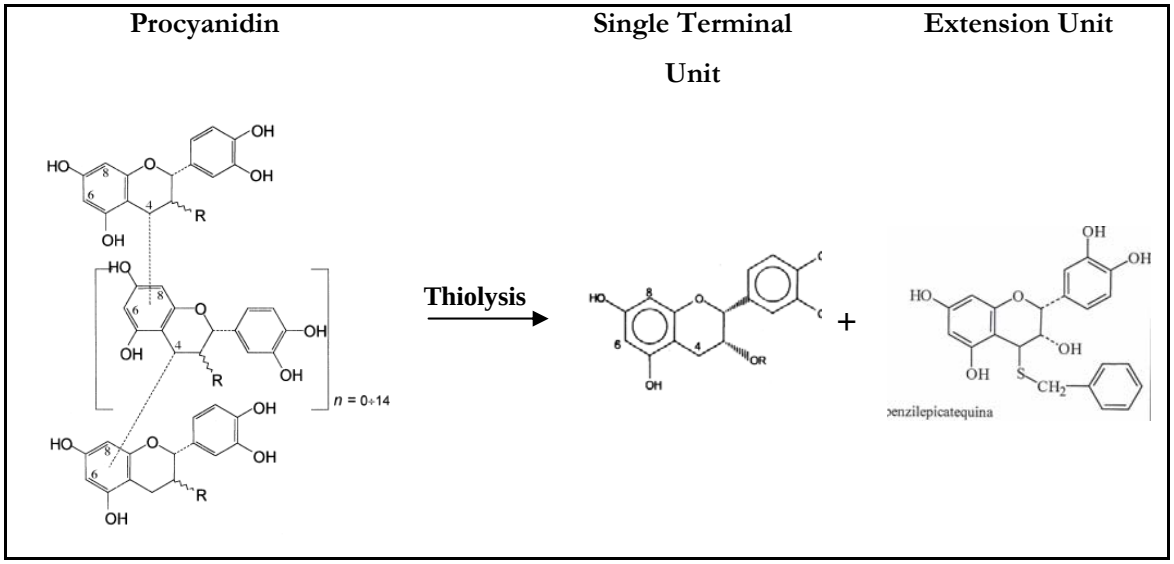


Figure 6 – Terminal and extension units after thiolysis of a procyanidin.

2.2 Angiotensin I-Converting Enzyme

In former section 2.1, several human health benefits of polyphenols were mentioned, namely, benefits in cardiovascular diseases. Several authors., [6,33];[34,35] showed that polyphenols can inhibit a particular enzyme: angiotensin I-converting enzyme (ACE), which inhibition has been proved to have beneficial effects in the treatment of several cardiovascular disorders [36]. The understanding of ACE action is important in the evaluation of the effect of polyphenolic compounds in the enzyme activity.

2.2.1. Structure and Properties of angiotensin I-converting enzyme

Angiotensin-I converting enzyme of mammals exists in two isoforms, one expressed in somatic tissue (sACE), and one in germinal cells in the males testes (gACE). The two forms differ in that the gACE has a single active site, whereas sACE has two active sites. The somatic protein is a translated tandem duplication. This duplicated structure produces a protein with two domains, the N-domain and the C-domain.

Both domains of sACE are functional, but with different biochemical properties. Their inhibitor affinity profiles differ. The requirement for chloride ions is very different between the two domains (C-domain specific activity dependent on chloride ion concentration). An inhibitor-specificity for each domain can be found. (for e.g., N-domain specific inhibitor, RXP 407 [37]). The two domains seem to have different functions. A preferential hydrolysis of angiotensin I and bradykinin by the C-domain [38] contrasts with a preferential processing by N-domain of other bioactives peptides for which it has a high affinity, such as the hematopoietic peptide.

Immunohistochemical assays [39] have shown that sACE is strongly expressed in many endothelial cells, especially in arterioles and small muscular arteries and in the normal capillary endothelial cells of the lung. Strong expression is also seen in the endothelial cells of the kidneys and small intestine and in a variety of neural cells in the brain. Expression of the second isoform, gACE is confined to differentiating male germinal cells.

The activity of the enzyme depends upon the presence of chloride ions. Chelating agents, sulphydryl compounds, heavy metals and certain peptides are inhibitory. The presence of zinc has been demonstrated in rabbit and canine enzymes [7] and its thought to be closely associated with human angiotensin converting enzyme. In phosphosaline buffer, serum ACE is most stable at pH 8.0-8.8 with an optimum pH of 8.3. Apart from variations in the glycoprotein content the human enzyme appears to be very similar if not identical to rabbit and canine enzymes.

ACE acts in two pathways, one is the formation of angiotensin II, in renin angiotensin system (RAS) and other is the cleavage of bradykinin (BK) in the Kinin System. Figure 7 shows ACE action in both systems.

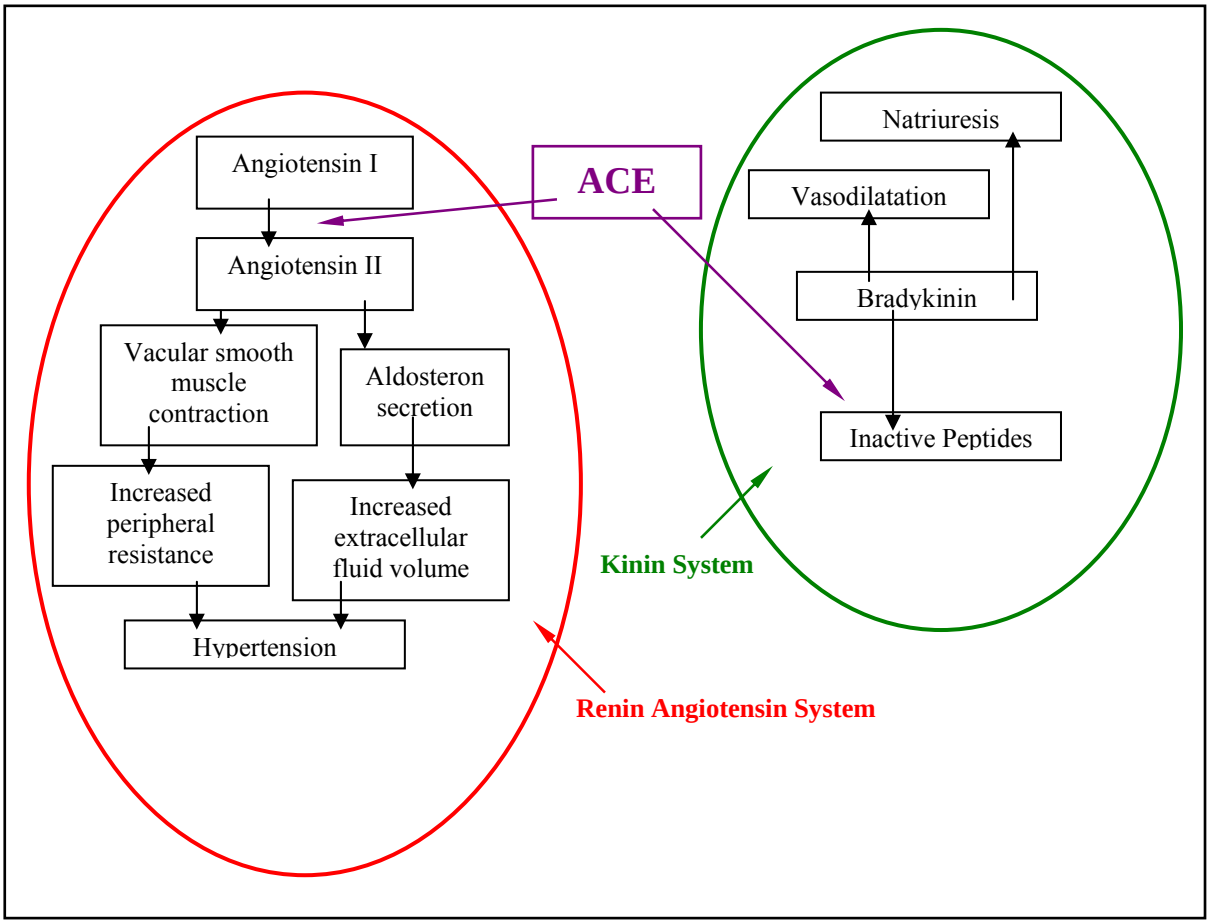


Figure 7 – ACE action in both Renin-Agiotensin and Kinin System

2.2.2. ACE action

2.2.2.1. Renin Angiotensin System

ACE inhibitors had been developed through the understanding of renin-angiotensin system. Although it has been discovered for more than 100 years, renin angiotensin system is yet widely studied. Its activation cascade includes several interactions enzyme-substrate that lead to the production of several biological active peptides. Nowadays, is considered that the renin angiotensin system does not possess only one final active product and that is not an exclusively circulatory system involved only in arterial pression regulation and hydroelectrolitic equilibrium. Several biologically active

angiotensinergics formed by diverse proteolytic pathways[1] characterize renin-angiotensin system as a system of multiple mediators [40] [41].

Renin-Angiotensin system has, therefore, two components-circulating and tissue. Whereas the first component participates acutely in maintaining adequate systemic hemoperfusion, the second component is chronically operative at the local tissue level. Despite these differences, both components share pathways for synthesis and degradation.

Figure 8 shows a simple representation of renin-angiotensin system. The angiotensinogenic substrate, (a glycoprotein released mainly in the liver) which is cleaved by renin, (an aspartyl protease produced in the kidney) releases the decapeptide angiotensin I (Ang I). Angiotensin-I converting enzyme (ACE), a zinc methaloprotease produced in the endothelium cells, mostly of the lungs, acts on the Ang I excluding two terminal aminoacids (His-Leu), releasing the octapeptide angiotensin II (Ang II), a powerful vasoconstrictor. Angiotensin II also stimulates the synthesis and release of aldosterone from adrenal cortex, which increases blood pressure by promoting sodium retention (and thereby water retention) in the distal tubules [42].

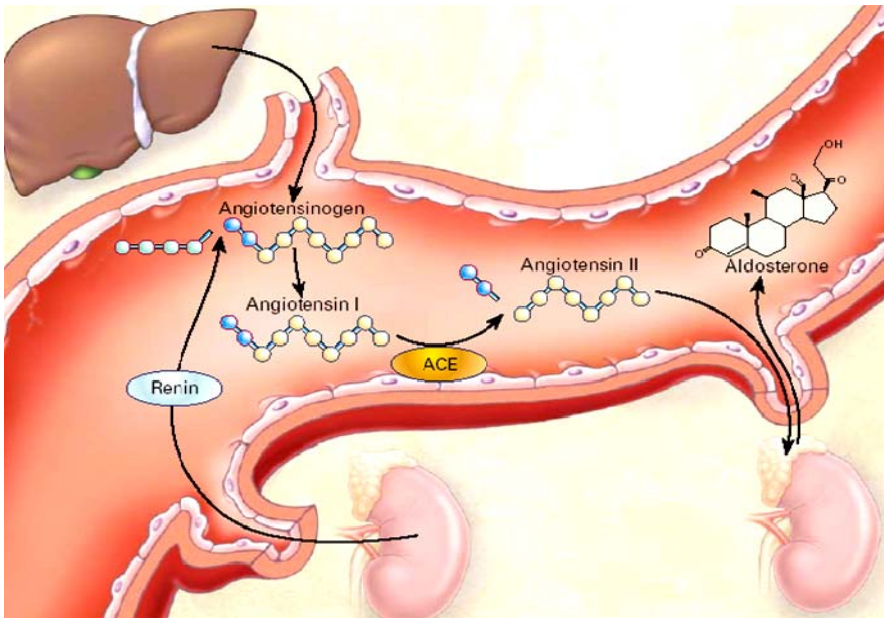


Figure 8 – ACE action in the Renin Angiotensin System

Angiotensin II and other angiotensinogens actions are mediated by multiple receptors located in plasmatic membrane of their target cells. Ang II receptors are present in a large number of organs and systems including heart, kidney, adrenal gland, pituitary gland, peripheric vasculature and central nervous system [43]. After interaction with the receptor, Ang II exerts diverse effects. Between systemic functions, show up arterial pressure maintenance and modulation, extracellular volume

[1] series of events controlled by proteases that occur in response to specific stimuli

control and renal and systemic circulation regulation. Kidney has a fundamental role as mediator of those effects, once Ang II acts in glomerular filtration and tubular reabsortion.

Moreover, alternative enzymatic pathways to the formation of Ang II; components and receptors of RAS; have been identified in others tissues, indicating a local formation and action of peptides of that system. That allows a modulation by paracrine[2] and autocrine[2] form of tissue functions of several organs, as heart, blood vessels, kidneys, brain, endocrine glands and central nervous system.

Figure 9 presents a schematic design of RAS and its regulation. The synthesis and renin release constitute the initial key of enzymatic cascade regulation which will product Ang II.

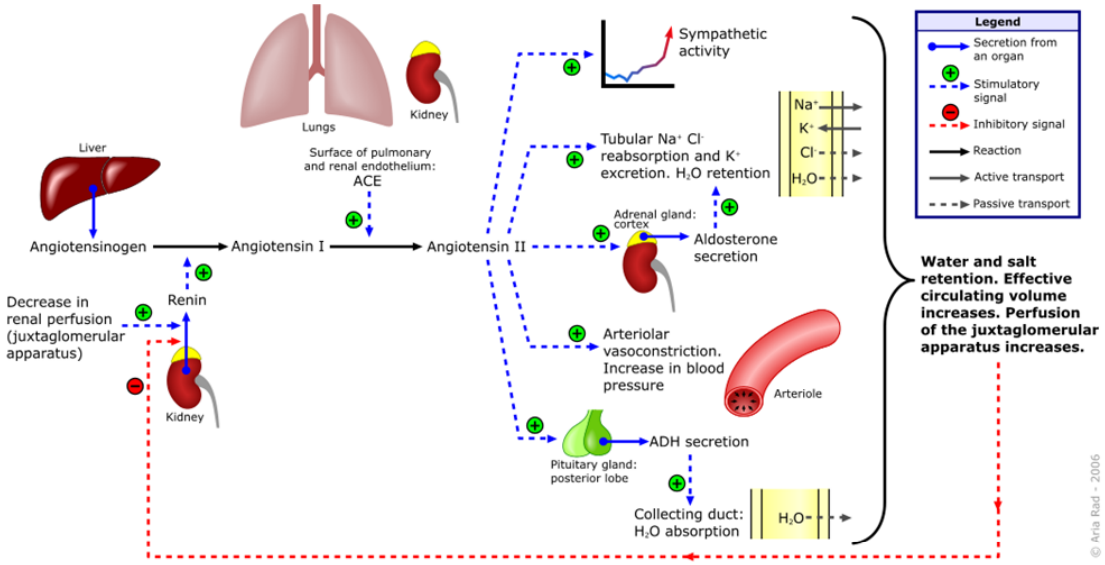


Figure 9 – Schematic representation of Renin Angiotensin System

Juxtaglomerulars cells produce the enzyme renin from pro-renin. Inside secretor glands, a pro-renin is converted in active rennin, which is released in blood stream as a response to diverse systemic and/or local stimulus [41].The systemic stimulation to renin release includes mainly the extracellular volume depletion and the decrease of systemic arterial pressure. After stimulation, renin is release into the blood stream and cleaves the angiotensinogen into angiotensin I which is relatively inert until is conversion in angiotensin II by angiotensin I-converting enzyme.

Angiotensin II formation leads to the i) increase in the sympathetic activity; ii) water and salt retention in distal tubules; iii) stimulation of synthesis and release of aldosterone from adrenal cortex

[2] type of hormone action in which a hormone binds to receptors on the cell type that produce it and affects their function; [3]type of hormone action in which hormone synthesized and released in endocrine cells binds to receptors in nearby cells and affects their function.

(that promotes water and salt retention); iv) arteriolar vasoconstriction, increasing blood pressure; and v) stimulation of ADH (antidiuretic hormone) that promotes water absorption in collecting duct. All this factors lead to water and salt retention, the increase of effective circulating volume and the increase of the perfusion of juxtaglomerular apparatus, which are the negative feedback for the release of renin.

2.2.2.2. .Kinin System

Although the major action of ACE is in Renin-Angiotensin System, this enzyme is also responsible for the increase of vasodilators substances as cinins and prostaglandins.

Kinin peptides have a broad spectrum of activities. Kinins are potent vasodilators and also promote diuresis and natriuresis. Recent studies in humans indicate a role for endogenous kinin peptides in the regulation of coronary vascular tone Kinins also participate in the cardinal features of inflammation, producing vasodilatation, vascular permeability and pain.

The B2 receptor subtype for bradykinin (BK) is widely distributed in mammalian tissue (endothelian and smooth muscle cells, afferent nerve endings, intestinal and renal epithelial cells) for which BK has high affinity and which physiological effects are vasodilatation and increase vascular permeability , hyperalgesia, natriuresis [44].

Though, Ang I was originally considered the main physiological substrate for ACE (K_m approximately $16\mu M$), because of its higher affinity (K_m approximately $0.18\mu M$) for BK, ACE could also be considered as a kininase (kininase II). As a peptidyl dipeptidase, ACE inactives BK by hydrolyzing two separate bonds on its C-terminal end. It removes sequentially the dipeptide Phe^8-Arg^9 and next cleaves the Phe^5-Ser^6 bond to generate the second dipeptide Ser^6-Pro^7, transforming BK into its inactive final $BK_{[1-5]}$ product [44].

The deleterious effects of ACE on the cardiovascular system were initially thought to be a consequence of the formation of Ang II, which initiates a cascade of events involving increased free radical production and vascular smooth muscle cell proliferation. However, as BK is much more readily hydrolyzed by ACE than Ang I, the hydrolysis of BK, may also contribute to this phenomenon [45].

2.2.3. ACE inhibitors

A wide spectrum of therapeutic intervention in hypertension was accomplished with the development of ACE inhibitors (hypertension, symptomatic or asymptomatic left ventricular systolic dysfunction, post-myocardial infaction, renal failure, and diabetic nephropathy [44]).

The action of ACE inhibitors, and the consequent reduction of the production of Ang II, leads to reduction of arterial pressure. ACE inhibitors not just reduce Ang II formation but are also responsible for the reduction of the sympathetic tono and the increase of vasodilators substances as cinins and prostanglandyns, which contribute to the hemodinamic effects and the efficacy of the anti-hypertensive agents.

ACE inhibitors, as captopril, enalapril, lisinopril and others, block competitively the conversion of Ang I to Ang II, decreasing the formation of Ang II. ACE has low specificity and participates also in the inactivation of cinins and other biological peptides. Although ACE inhibitors show a high efficacy, the lack of specificity leads to the research of alternatives to this compounds [46]. Some of the side adverse effects as dry cough, rash, lost of taste and pro-inflamatory effects attributed to ACE inhibitors emphasize the research for alternatives. Polyhenolic compounds, namely procyanidins, interact significantly with biologic system like enzyme-inhibition system and been proven to inhibit Angiotensin I-Converting Enzyme [6,33]. The effect of these natural compounds on ACE could contribute to the decreased risk for cardiovascular diseases observed in populations that consume high amounts of foods rich flavonoids.

2.2.4 Methods for measuring angiotensin I-converting enzyme activity

A number of different methods for evaluating angiotensin converting enzyme are available.

Cushman and Cheung, [47], described a method for assaying the enzyme activity in rabbit lung extracts and measured the activity in terms of rate of release of hippurate from a substrate analogue hippuryl-L-histidyl-L-leucine, which substitutes for angiotensin I. The substrate and the enzyme are incubated under controlled conditions. The reaction is terminated by acidification, and after separating the unhydrolysed substrate by extraction with ethyl acetate, hippurate is measured spectrophotometrically by determining its absorbance at 228 nm. However this method is difficult to control since all traces of ethyl acetate must be removed by evaporation for this substance absorbs strongly at 228 nm and therefore interferes with the assay. Chiknas et al. [48] described a modification of Chusman and Cheung's method in which high pressure chromatography is used to measure the hippuric acid end product. This procedure overcomes a number of problems encountered with the

spectrophotometric assay. Friedland and Silverstein[49], described a sensitive and reproducible fluorimetric assay using hippuryl-L-histidyl-L-leucine as substrate in which the rate of production of L-histidyl-L-leucine is quantified spectrofluorometrically by the formation of a fluorescent adduct with *O*-phthaldialdehyde. This method is highly sensitive. Friedland and Silverstein [49] found a highly correlation between measured serum ACE activity determined with angiotensin I compared with the analogue substrate hippuryl-L-histidyl-L- leucine, (r=0.93).

Since these methods evaluate the activity of an enzyme, kinetic parameters must also be determined. These parameters allow us to define the behaviour of the enzyme in the experiment conditions and work in preferential conditions

As enzyme-catalysed reactions are saturable, their rate of catalysis does not show a linear response to increasing substrate. If the initial rate of the reaction is measured over a range of substrate concentrations (denoted as $[S]$), the reaction rate (V_0) increases as $[S]$ increases, However, as $[S]$ gets higher, the enzyme becomes saturated with substrate and the rate reaches V_{max}, the enzyme's maximum rate. This can be represented by Michaelis-Menten kinetic model of a single-substrate reaction as showed in Figure 10.

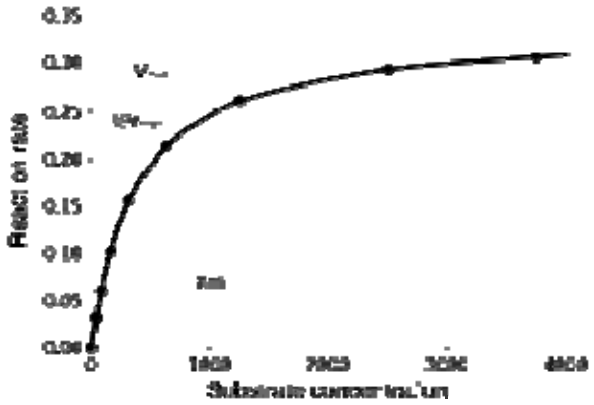


Figure 10 – Substrate concentration effect on the reaction rate of an enzymatic reaction.

Michaelis-Menten equation (Equation [1]) is the velocity equation to a catalytic reaction with a single substrate. It's a qualitative expression between initial velocity, V_0, maximal initial velocity, V_{max}, and the initial concentration of substrate $[S]$, all related by Michaelis-Menten constant, K_m.

$$V_0 = \frac{V_{max} \cdot [S]}{K_m + [S]}, \qquad \textbf{Equation [1]}$$

Michaelis-Menten constant is a dynamic constant, which express relation between real concentrations in stationary state instead of equilibrium concentrations.
An important numeric relation is obtained from Michaelis-Menten equation in the special case when V_0 is exactly half of $V_{\max}$ (Equation [2]).

$$K_m = [S] \rightarrow V_0 = \frac{1}{2} V_{\max}, \qquad \textbf{Equation [2]}$$

K_m is equivalent to the substrate concentration in which V_0 is equal to half of $V_{\max}$, and indicates the "affinity" of an enzyme to the substrate. As minor is the K_m value, higher will be the affinity of the enzyme to the substrate.

Chapter 3 - Experimental Section

Chapter 3 describes the methodology in the different steps developed in this work. Description is supported by several schemes to provide a simplified visualization of experimental procedures. Chapter is divided in two main sections:

i) Grape seed treatment extraction and characterization (oil and polyphenolic compounds);

ii) Evaluation of Angiotensin I-converting enzyme activity in the presence or absence of polyphenolic fractions. Captopril and pure monomers were also tested.

3.1. Grape seed

Grape seed extraction (oil and phenolic compounds) and characterization was performed with the help of several methods and equipments. Methods differ concerning the target of extraction or characterization. In some cases, before characterization, samples were submitted to chemical processes for a better detection of samples constituents.

Grape seeds, (*Vitis vinifera* L. white variety. 'Chardonnay'), grown during 2001 season in Bairrada Apellation experimental vineyard (Estação Vitivinícola da Bairrada, Anadia, Portugal), were harvested at technological maturity (September, 2002) and collected during transfer of the must in wine fermentation. Seeds wash treatment and sieving was performed as described by Passos et al.[50]. Seeds were separated from pulp and skins, by decantation and sieving, and were intensely washed. A first wash, to remove immature grains floating at water surface, was followed by several washes with water (200g/L) under gentle stirring with a magnetic bar. Washes were carried out at 4ºC during a minimum of three days, with two water exchange per day, until minimum constant turbidity was observed. Seeds were finally washed with ethanol, air dried at room temperature, and stored at 4ºC until use. After immersion into liquid nitrogen, seeds were milled on a domestic coffee mill and classified with a sifter with different size sieves. An average particle diameter $\overline{d}_p$ mm was calculated by Sauter's equation [51] to a set of fractions within [0.5;1.4]:

$$\overline{d}_p = \frac{m_t}{\sum_{i=1}^{k} \frac{m_i}{d_{p_i}}}$$ **Equation [3]**

3.1.1. Grape Seed Oil

3.1.1.1. Grape Seed Oil Extraction

Extraction of grape seed oil was carried out with a conventional soxhlet aparattus (Figure 11). Seeds were weighted and placed in a $23 \times 10 mm$ cartridge ($\approx 10 g$). The cartridge was putted in the soxhlet thimble holder ($50 mL$ capacity) and the apparatus was set with 150 mL of n-hexane in the solvent flask. Extraction started setting heating bath temperature to 80ºC and took place during 4 hours. After extraction, solvent was evaporated and solute concentrated under vacuum at 30ºC. To ensure that the oil carries no water, the extracted samples were passed over sodium sulphate anhydrous under vacuum in a G1 sintered glass filter, and evaporated in a rotary evaporator also under vacuum at 30ºC. The oil was then transferred to speed-vacuum tubes and dried by centrifugal evaporation. The mass of extracted oil was determined gravimetrically after solvent evaporation.

Figure 11 – Oil extraction from grape seeds with soxhlet apparatus

3.1.1.2. Grape Seed Oil Characterization

Oil characterization was performed by gas chromatography with flame ionization dectection (GC-FID). Previously transesterification of fatty acid methyl esters was made. Transesterification was carried adapting IUPAC methodology without heating, (preventing the risk of polyunsaturated acids decomposition). An internal standard, heptadecanoate methyl ester (C17), was used during transesterification process. An oil sample of $100mg$ was dissolved in $1mL$ of n-hexane and then added $4mL$ of internal standard solution ($750mg/mL$). A volume of $200\mu L$ of methanolic KOH solution ($2M$) was added to the vial with the previous mixture. The vial was sealed and vigorously mixed for 30 seconds in a vortex shaker. It was then added $2mL$ of saturated sodium chloride solution, to promote "Salting Out" (polyunsaturated acids pass from the aqueous phase to the organic phase). The sample was then submitted to centrifugation at $2000rpm$, during 5 min. Finally, $1mL$ of the organic phase was then transferred to another vial and used to GC-FID analysis.

GC-FID was used to separate and detect polyunsaturated acids. A Perkin Elmer Clarus 400 Gas Chromatograph, equipped with 30 m x 0.32 mm (i.d.) DB-FFAP fused silica capillary column (J&W Scientific Inc., Folsom, CA, USA) and a flame ionization detector, was used Split injection mode used with a ratio of 20:1 (5 min). The GC injection port was programmed at 245ºC, whereas the detector at 250ºC. Oven temperature was programmed from 75 to 155ºC at 15°C /min, from 155 to 180ºC at 3ºC/min, from 180 to 220ºC at 40ºC/min, and held isotermic for 3min. Carrier gas is hydrogen (50 mL/min). All measurements were made with, at least, three replicates, each replicate representing the analysis of one different aliquot (100mg) of grape seed oil.

3.1.2. Grape Seeds Phenolic Compounds

3.1.2.1. Grape Seed Phenolic Extraction

Phenolic content of grape seeds was extracted following Guyot et al. [52] methodology and adapted by Passos et al. [50]. Figure 13 represents extraction procedure. After oil extraction with *n*-hexane, grape seeds left dried at room temperature. Phenolic compounds were then extracted with a three times treatment (15 minutes each, at room temperature) with methanol containing 5% acetic acid (100 g seed powder/L MeOH). The acetic acid avoids the oxidation of compounds during extraction. The phenolic extract with methanol was filtered through, first a G1 and then a G3 sintered glass filter. After blending the three extracts, the solute was concentrated under vacuum at 40ºC, with several additions of water to assure the complete removal of methanol and acetic acid (Figure 12). Further, the seed powder product of methanol extraction is treated with a mixture of acetone/water (2:3) and 5% acetic acid (15 minutes at room temperature). The extraction with acetone/water mixture was repeated three times and the phenolic extract was treated in the same procedure as phenolic extract from methanol. Solute was frozen and lyophilized in a Virtis Sentry 5L. Samples were stored and labelled with the designation WM for white grape extracted with methanol and WA for white grape extracted with acetone/water mixture.

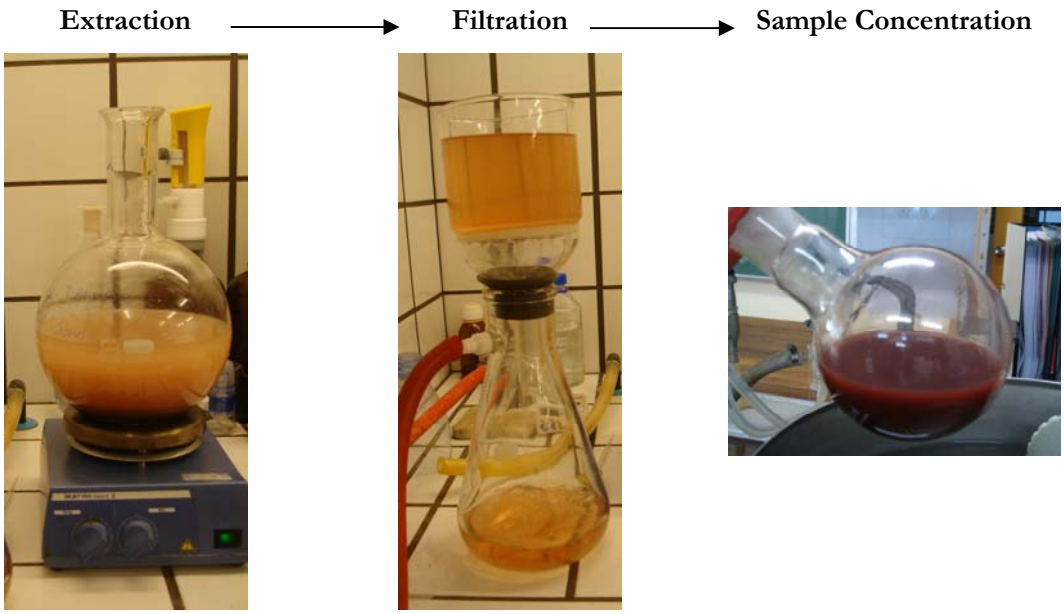


Figure 12 – Phenolic extraction of grape seed followed by filtration and concentration of the sample

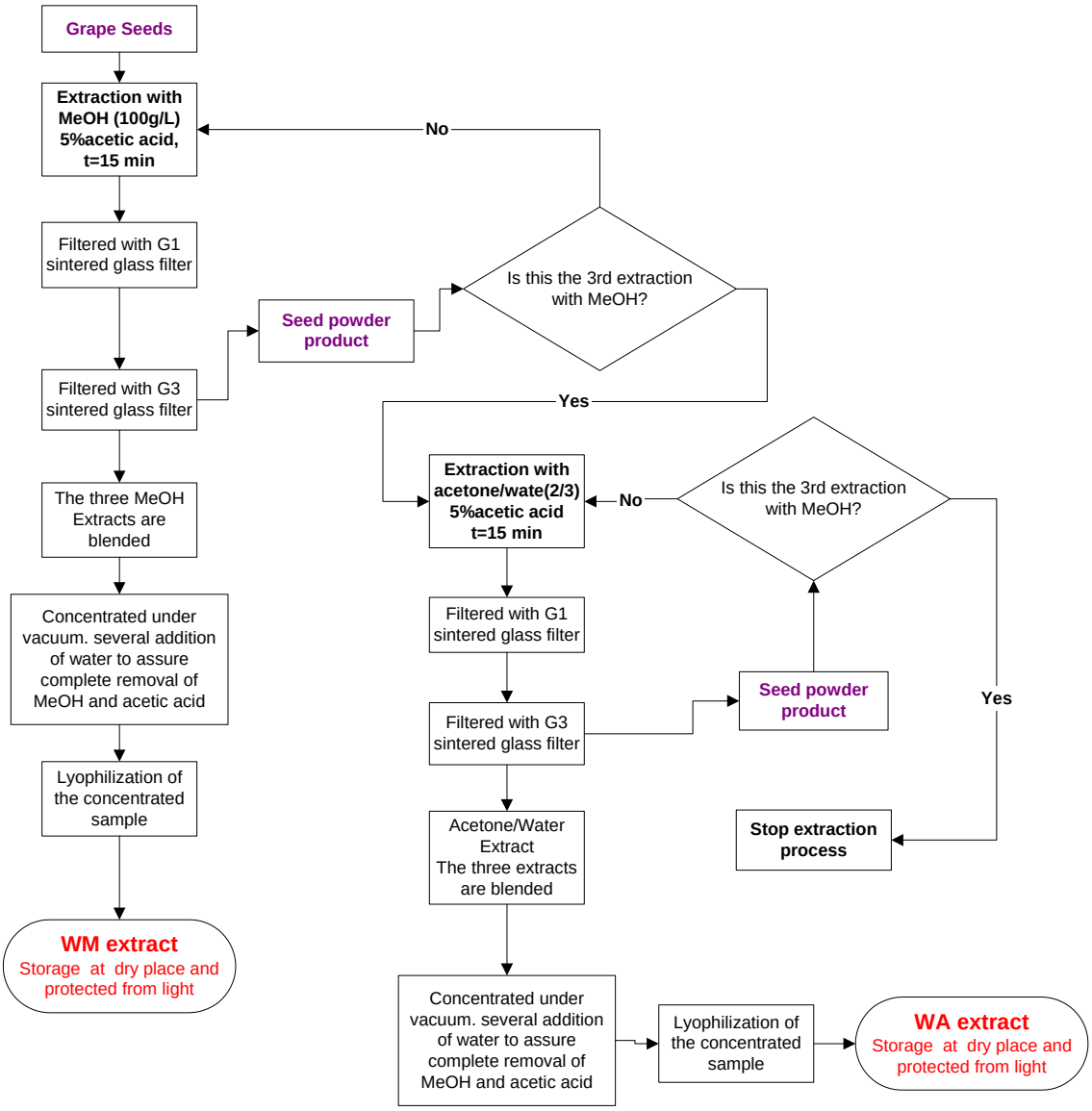


Figure 13 – Grape seed extraction methodology

3.1.2.2. Grape Seed Phenolic Extract fractionation

(Fractionation and characterization was executed by Passos et al.)

The methanol phenolic extract and the acetone/water phenolic extract from white seeds were fractionated according to the methanol/chloroform graded precipitations proposed by Saucier et al. [53] and adapted by Passos et al. [50]. Procedure is summarised in Figure 14. The phenolic extract powder (10 g/L) was dissolved in water containing 5% acetic acid and the undissolved material (F_0) was removed by centrifugation (Centrifuge 3K30, Sigma, St. Louis, MO, USA). The supernatant was then submitted three times to a liquid-liquid extraction with ethyl acetate, using a supernatant/ethyl acetate ratio of 3:2 (v/v), resulting in an organic phase (F_1) and an aqueous phase (F_2). The F_1 solution

(phenolic extract in organic phase) was concentrated and loaded into a C18 solid phase extraction column (SPE-C18, SPE, Supelco-Discovery – 5g) by eluting with diethyl ether followed by methanol, allowing the obtaining of fractions $F_{1.1}$ and $F_{1.2}$, respectively. Elution of $F_{1.1}$ and $F_{1.2}$ started and stopped when the presence or absence, respectively, of polyphenolics was detected at 280 nm.

The F_2 solution (phenolic extract in aqueous phase) was evaporated to dryness and redissolved in methanol (10 g/L). The undissolved material was removed by centrifugation (15000 rpm; 15 min; 10ºC) and the supernatant was submitted to successive additions of chloroform until a new precipitate was formed. Each addition was processed overnight, with stirring, at 4ºC. The concentrations of chloroform in which the different fractions precipitated are given in Table 2.

Table 2 – Percentage of chloroform/methanol used to obtain fractions from $F_{2.0}$.

Fraction	**% of Chloroform in methanol**
$F_{2.1}$	29
$F_{2.2}$	52
$F_{2.3}$	66
$F_{2.4}$	73
$F_{2.5}$	79
$F_{2.6}$	84

The precipitate was then collected by centrifugation, dissolved in water and rotary-evaporated with several additions of water to completely remove the organic solvents. Sample was then frozen and lyophilized in a Virtis Sentry 5L. Samples were stored.

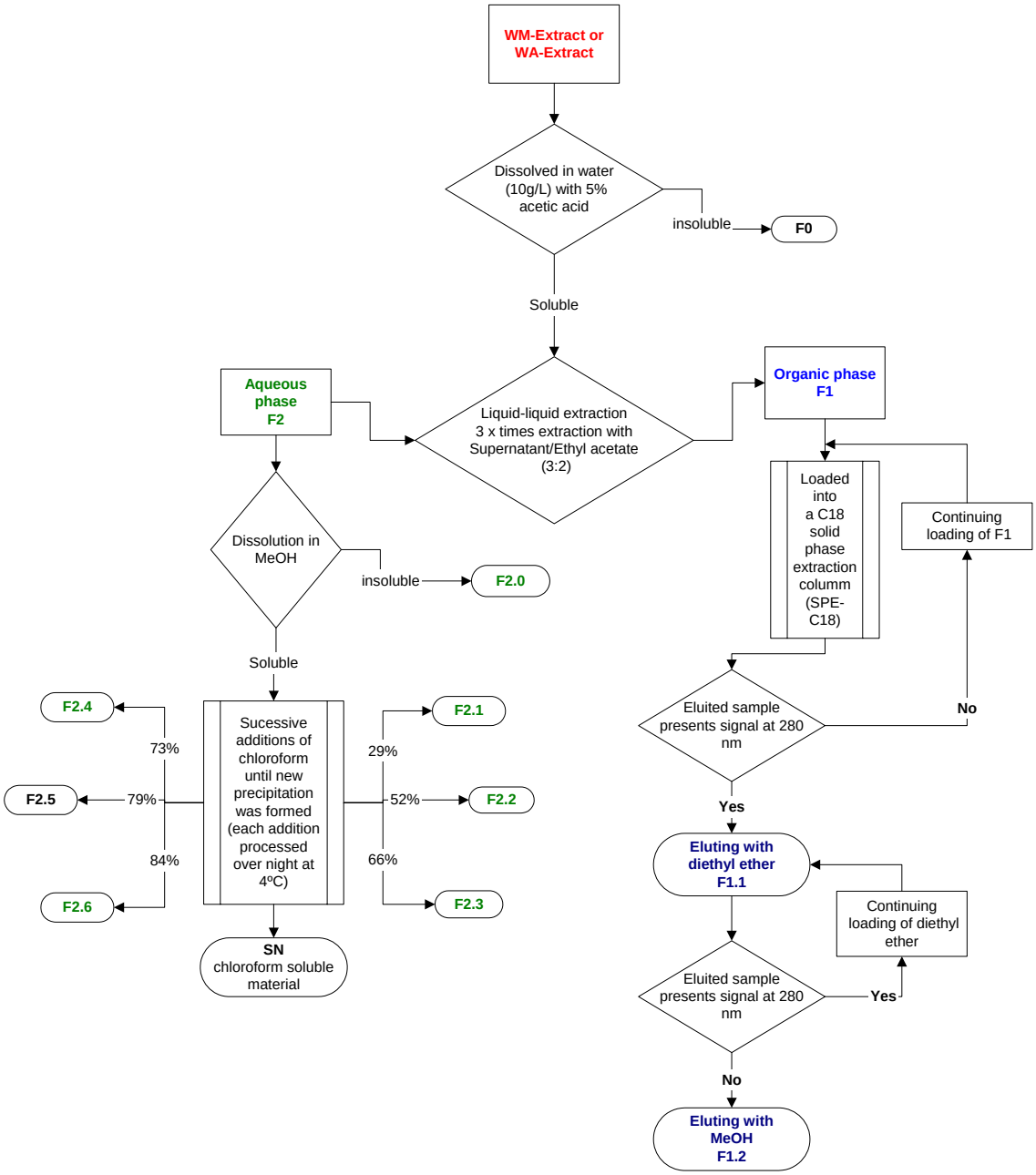


Figure 14 – Fractionation methodology of phenolic extract

3.1.2.3. Grape seed phenolic extract characterization

Grape seed phenolic extracts were characterized by HPLC. The analysis with HPLC that provides identification and quantification of extension and terminal units of polymerized polyphenolics requires a pre-treatment before sample injection. Thiolysis was carried out according to the methodology described by Guyot et al. [52]. A 2 mg/mL suspension was prepared by sonicating the residue in MeOH acidified by HCl (1.66%, v/v); $100\,\mu L$ of the suspension was introduced into a glass

bulb together with $100\mu L$ of phenylmethane-thiol (5% in MeOH). After sealing, the reaction was carried out at 25ºC, for 24 hours and then $10\mu L$ are directly injected on to the HPLC system. HPLC analysis followed the conditions described by Peng et al. [54] and adapted by Passos et al.[50].

3.2. ACE activity

Extraction and characterization of oil and phenolic content of grape seeds, was followed by the evaluation of the capability of phenolic extract to inhibit ACE and therefore their benefits in cardiovascular diseases. The ACE activity protocol was established after some kinetics determinations. Inhibitors were then tested: Polyphenolic rich-extract, a powerful ACE inhibitor and pure monomers (which are found in the constituents of polyphenolic extracts), were tested.

The activity of ACE was determined first in the absence of the inhibitors, after in the presence of monomers and lastly in the presence of fraction of grape seed phenolic extracts. All determinations were analysed following the same methodology, proposed by Friedland and Silverstein[49], and adapted by Tsutsumi[35]. ACE activity was assayed by fluorometric determination of the amount of histidyl-leucine (His-Leu) released from a substrate, hippuryl-histidyl-leucine (Hip-His-Leu), in the present of ACE, as described in Figure 15. A reaction medium of 100 mM HEPES buffer (pH 8.3) containing 300 mM NaC1, was employed to prepare all compounds. For the determination of ACE activity, Hip-His-Leu substrate was used to initiate all reactions after pre-incubation of ACE (0.75mU) at 37°C, for 3 min. When ACE activity was determined in the presence of inhibitor, the inhibitor was pre-incubated for 15 min with the enzyme before adding the substrate. The enzyme reaction was carried out at 37°C, for 30 min, in a final volume of 150 ul and then terminated with 30 ul of 1N sodium hydroxide. The His-Leu product in the reaction mixture was estimated following *o*-phthaldialdehyde treatment. To the reaction mixture, 10 ul of 0.2% *o*-phthaldialdehyde in methanol was added to form the *o*-phthaldialdehyde condensation product of His-Leu. The mixture was incubated at 0°C for 15 min in the dark; then 30 ul of 1.5M perchloric acid was added for neutralization. The fluorescence of the *o*-phthaldialdehyde condensation product of His-Leu was determined with a spectrophotofluorometer Spectramax Gemini (Molecular Devices, Sunnyvale, CA, USA), (360nm excitation, 480nm emission wavelength, cutoff of 420 nm).

A standard curve (His-Leu) was prepared, in 100 mM Hepes, 300 mM Nacl buffer, pH 8.3: 0; 0.625; 1.25; 2.5; 5 mM, for each assay. All assays including the fluorometric controls were individually repeated seven times.

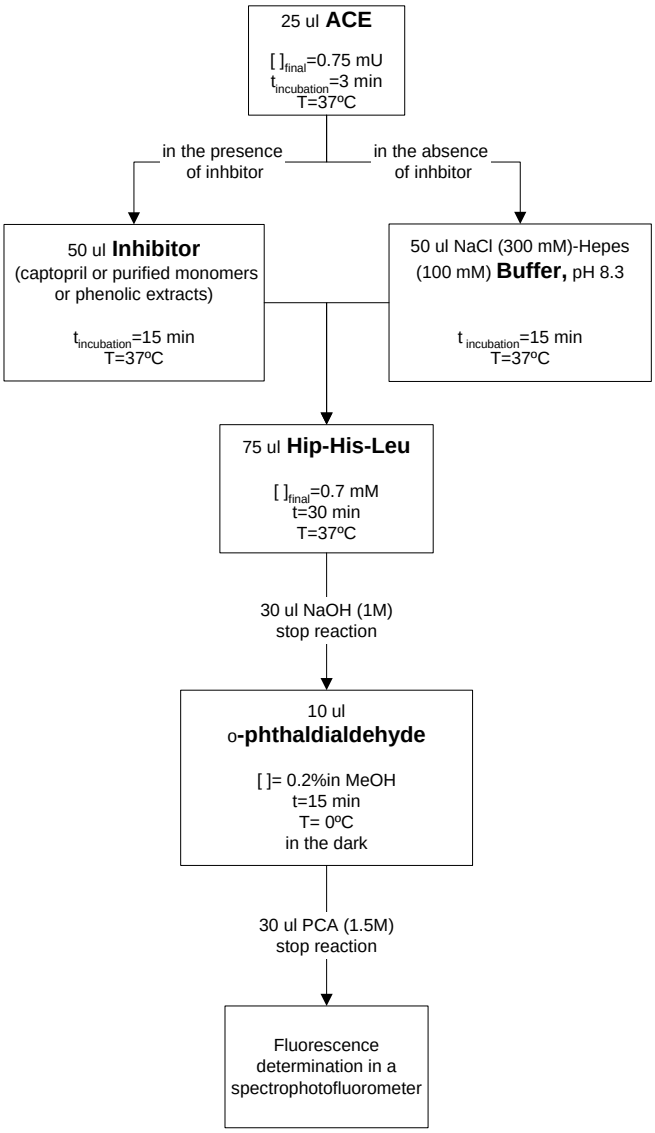


Figure 15 – ACE activity methodology

3.2.1. Determination of Kinetic parameters of ACE

3.2.1.1. Determination of Km

For the determination of Km, a curve of ACE activity in relation to substrate concentration was draw. ACE activity was determined as described above. Hip-His-Leu substrate, 0.05-5 mM, was used to initiate reaction after pre-incubation of ACE (0.75mU) at 37°C, for 3 min. Km was determined adjusting curves to the Michaelis-Menten equation.

3.2.1.2. Time course determination

The same methodology described above was used to determine the time course of this reaction. Substrate and enzyme were incubated for 15, 30, 45 and 60 min. The substrate concentration was fixed, 3 mM (saturated concentration) and the remaining experimental conditions were maintained.

3.2.2. Evaluation of the ACE Activity

3.2.2.1. In the absence of the inhibitor

The ACE activity in the absence of inhibitor was assayed as described above. Substrate concentration was chosen and a 0.7 mM of Hip-His-Leu was used in all experiences performed. After 3 min of enzyme incubation, 50 ul of NaCl-Hepes buffer was added and left incubating during 15 min to mimic inhibitors addition. Remain experimental conditions were maintained.

3.2.2.1. In the presence of inhibitors

For evaluation of inhibitory effect in the ACE activity, several compounds were tested. Primarily, by captopril which is a well known inhibitor of ACE, followed by pure flavanol monomers and lastly 4 fractions of grape seed polyphenolic extracts. Inhibitors were added after ACE incubation during 15 min (Figure 16). Remaining methodology follows what was previously described.

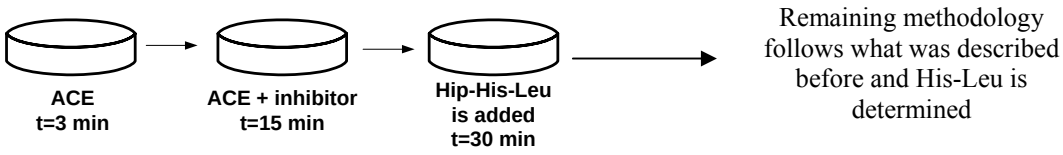


Figure 16 – Procedure with Inhibitors

- **Captopril**

Following the same ACE activity protocol, captopril was tested. This positive control was performed with captopril form 0 to 50 nM. After 3 min enzyme incubation, 50 ul of Captopril was added and incubated with the enzyme for 15 min. Remain experimental conditions were maintained.

- **In the presence of pure flavanol monomers**

Following the same ACE activity protocol, pure monomers compounds, (+)-catechin, (-)-epicatechin, (+)-catechin-O-gallate, (-)-epigallocatechin were tested. After 3 min enzyme incubation, the monomers (0-100 uM) were added and incubated with the enzyme for 15 min. Remain experimental conditions were maintained.

- **In the presence of Grape seed phenolic extracts**

Following the same ACE activity protocol, fractions of phenolic extract, WM-$F_{2.2}$, WM-$F_{2.4}$, WM-$F_{2.6}$ and WA-$F_{2.2}$ were tested. After 3 min enzyme incubation, grape seed extracts (0-30 mg extract/L) were added and incubated with the enzyme for 15 min. Remain experimental conditions were maintained.

Chapter 4 - Results and Discussion

Results will be presented in this chapter. Discussion of results will be taken through the chapter following results appearance. This chapter was divided in two main sections:

i) Grape seed oil and phenolic content extraction and characterization

ii) Angiotensin I-converting enzyme activity in the absence and presence of phenolic fractions, captopril and pure monomers.

4.1. Grape Seed

Two important components of grape seeds were studied: grape seed oil and polyphenolic compounds. Extraction was accomplished to obtain these two constituents and characterization was made with specific methods and using pre-treatment of the samples when was required.

Before extraction grape seed diameter was determined. For the particle diameter determination, sieves with different particle sizes were weight and the average was determined with Sauter's equation (Equation [3]). An average diameter of 0.7 mm was obtained. In this study only one average diameter was use for extraction since the objective was to characterize the grape seed oil and not determine optimal extraction conditions. Nevertheless, the particular diameter is an important step to the subsequently extraction. Fiori [55] describes a clear influence of the particle size in the oil extraction yield. The smaller the particle the greater the final yield. A small change in particle size (diameters of 0.49 and 0.51 mm) results in significant change in the extraction curve.

4.1.1. Grape seed Oil

4.1.1.1. Extraction Yield

The n-hexane extraction of grape seed with conventional soxhlet apparatus results in 11.6% of oil extract (average of three assays). Extraction yield was calculated as described in Equation [4]. Grape seed dried mass (GS_{DM}) was determined taking into account previously determination of moisture content in raw material. Moisture was determined weighting a sample of grape seed at room conditions and weighting the same sample after leaving seeds over night at 105ºC. Oil extract dried mass (O_{DM}) was determined weighting the mass of oil resulting from soxhlet extraction and after all hexane and water removable.

$$Oil(\%) = \frac{O_{DM}}{GS_{DM}} \times 100 \qquad \textbf{Equation [4]}$$

Table 3 shows the determination parameters for oil extraction yield. The % of oil obtained, 11.6%, is in accordance to other authors results, 10-16 % [8,12,13,56].

Table 3 – Percentage of grape seed oil in each assay tested.

Assay	Grape Seed Mass (GS_M) (mg)	Moisture (%)	Grape Seed Dried Mass (GS_{DM}) (mg)	Oil Dried Mass (O_{DM}) (mg)	Grape Seeds Oil (%)
A1	10.87	5.91	10.22	0.57	11.15
A2	9.64	5.91	9.07	1.11	12.30
A3	9.91	5.92	9.32	1.06	11.32

The extraction yield depends of several extractions conditions, particle size, time extraction, solvent and method used. In these essays no conditions were tested. Assuming for particle size, extraction time, solvent choice and the method used, those already determined by the Passos et al. The aim of this work, as said before, was to characterize grape seed oil and not to determine optimal conditions for extraction.

4.1.1.2. Grape Seed Oil Characterization

To characterize grape seed oil a derivatization of the oil sample was required. Using transesterification, a process that breaks triglycerides linkages and leads to the formation of the esterified acid, fatty methyl esters can be identified. An internal standard, C17, was include in the oil sample and went through the same transesterification process before injection in GC-FID. Three replicates of the grape seed oil extracted in soxhlet apparatus were injected. Figure 17 shows the highlight of the chromatogram of one replicate. Peaks are identified in the Figure 17.

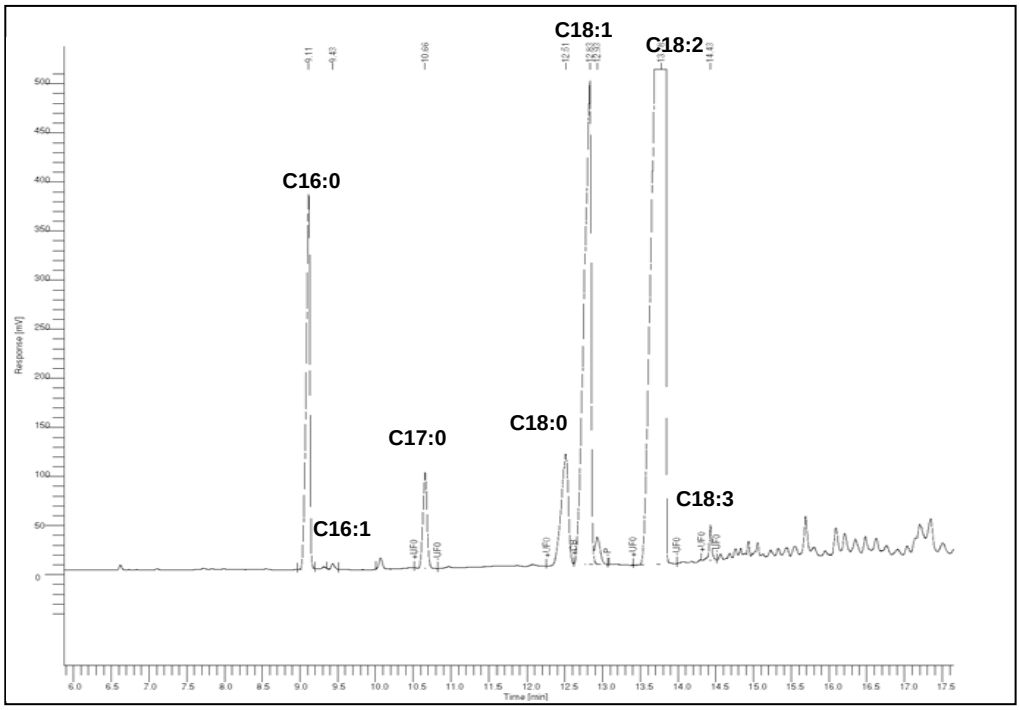


Figure 17 – Highlight of the chromatogram of methyl esters of grape seed

The order of peak appearance and peak identification was determined with injection of standards of the methyl esters. In the chromatogram, the palmitic acid (C16:0) was the first fatty methyl ester appearing followed by C17:0 (internal standard); palmitoleic acid (C16:1), stearic acid (C18:0); oleic acid (C18:1); linoleic acid (C18:2) and linolenic acid (C18:3). Using standard response and respective response factors, quantification of fatty acids present in the sample was possible.

Figure 18 provides the % of fatty acids present in the grape seed oil sample grape seed oil. Composition of grape seed oil obtained was almost the same that for a commercial grape seed oil. The same composition indicates that the extraction with soxlhet and the conditions used were good operating conditions and do not interfere with oil quality.

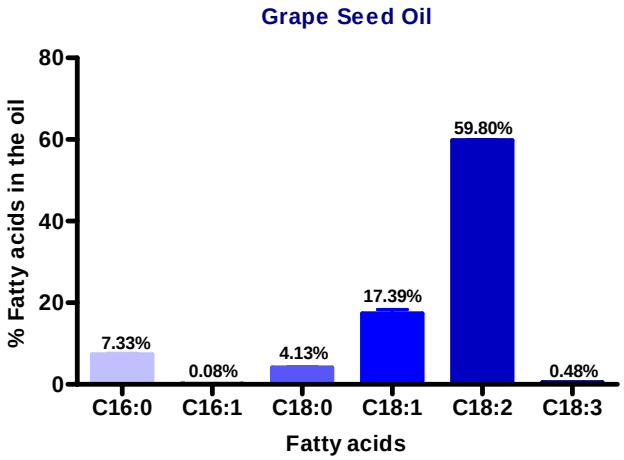


Figure 18 – Characterization of grape seed oil extract.

Regarding grape seed oil composition, the main fatty acid present is linoleic acid (60%, 18:2n-6); followed by oleic acid (17%, 18:1n-9), which totalizes 77% of polyunsaturated fatty acids. The remaining compounds include the saturated palmitic acid (7%, 16:0), stearic acid (4%, 18:0) and linolenic acid (0.5%, 18:3n-3). Only traces of palmitoleic acid (16:1n-9) – less than 0.1% – were found. High levels of unsaturation ensure grape seed oil as a high quality oil. High levels of unsaturated acids play an important role in lowering high blood cholesterol and also in the treatment of atheroscleoris [8].

In fact, the composition of fatty acids in grape seed oil makes this oil, an edible oil with high quality as other nutrition oils. The content of saturated fatty acids (10%) is only a little higher than of rappseed oil but comparable to most of other commonly used edible vegetable oils. The content of linolenic acid, clearly below 1 %, is comparable to sunflower oil as well for linoleic acid and oleic acid, 48-74% and 14-40%, respectively.

4.1.2. Grape Seeds Polyphenolic Compounds

As for oil extract, the polyphenolic content in grape seeds was obtained. Since grape seed polyphenolic extracts contains a large spectrum of procyanidins with differents degrees of polymerization a fractionation process was required. After this process, different fractions were characterized. Characterization was made by High Perfomance Liquid Chromatography (HPLC). HPLC characterization was performed by Passos et al, [50] and results were kindly provided to this work for further discussion. For the characterization with HPLC thiolysis of samples was required.

4.1.2.1. Extraction Yield

Polyhenolic compounds of grape seed were obtained with a series of two extractions. Grape seed free of oil (previously treated with hexane) was extracted first with methanol and after with the mixture of acetone/water. Guyot et al. [52] described the first extraction with methanol responsible for the extraction polyphenolic compounds of low molecular weight. The extraction with acetone/water would remove polymeric procyanidins. However, during the extraction of grape seeds, the first extraction with methanol seems to be responsible already for the extraction of polymeric procyanidins, as the fractions characterization will show. The yield of the extraction was determined. Grape seeds presented 25.5% of an extract rich in phenolic compounds. Part of the extract are probably other compounds like sugars and organic acids that are also present in grape seeds and that are also extractable. Other authors refer that polyphenolic compounds in grape seeds correspond to 9 % [8]

and refer 20 % of others extractable compounds. Fuleki and Ricardo da Silva [26] refer a percentage range of 0.80-17.7%. The difference on the content of polyphenolic compounds can be explained by the different composition of different varieties of grapes, since several factors, as environmental and viticulture, can influence polyphenolic composition of grapes. The operating conditions of extraction process can also influence phenolic composition. In this case the main objective of the work was to obtain a considerable amount of polyphenolic extract that could permit enough material for further characterization and inhibition tests. In fact, extraction was carried in a batch process, without huge concern in optimize extraction conditions and consequent yield.

4.1.2.2. Grape Seed Phenolic Compounds Characterization

Polyphenolic extract from grape seeds was fractionated, in accordance to the solubility of the polyphenolic content in a binary mixture of chloroform/methanol. Several fractions of grape seeds were obtained. Fractionation procedure was executed by Passos et al.

Since just four fractions of phenolic extract (WM-F2.2; WM-F2.4, WM-F2.6, WA-F2.2) were used in follow work (ACE inhibition study), only these fractions yield are shown. Table 3 presents the results from fractions yield. Values should be considered in relation to total mass in F2. The successive precipitations achieved, by the addition of different proportions of the binary mixture of chloroform/methanol, permit separate the total polyphenolic extract into fractions, divided by their degree of polymerization. HPLC analysis permits to identify terminal and extension units of (+)-catechin, (-)-epicatechin and (-)-epicatechin-3-*O*-gallate, obtained after thiolysis degradation. Standards were also injected. A chromatogram with the results of one injection of fraction WM-F2.2 is showed in Figure 19. Peaks are identified in the Figure 19. Results were kindly provide from Passos et al. [57].

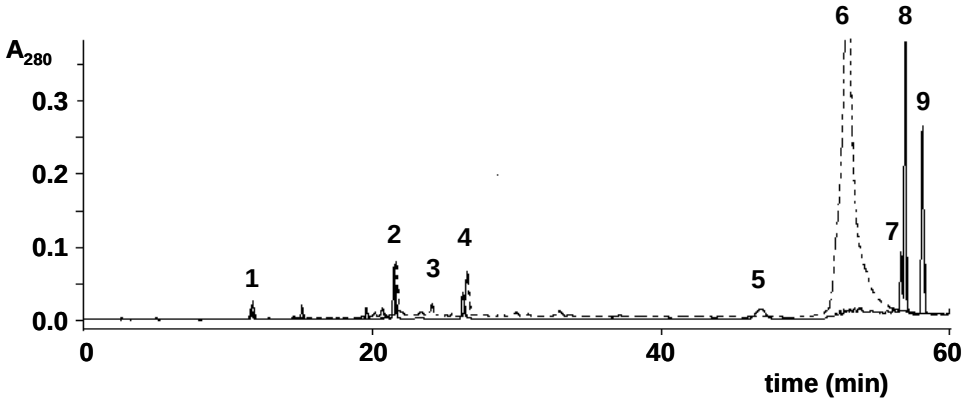


Figure 19 – Chromatogram of fraction WM-F2.2

The elution of sample compounds was first gallic acid (1), followed by catechin (2); procyanidin B2 (3); epicatechin (4); epicatechin-*O*-gallate (5); procyanidins (6); catechin benzylthiother (7); epicatechinBenzylthioether (8); epicatechin-*O*-gallate benzylthioether (9).

Extension and terminal units were distinguished by the presence of thiol group. Only extension units present a thiol group.

DPn was calculated after HPLC identification by Equation [5].

$$DPn = \frac{termial\_unit(flavanol) + extensionu\_unit(flavanol)}{ter\min al\_unit(flavanol)} \qquad \textbf{Equation [5]}$$

Tabela 4 shows the extension and terminal units of each sample tested as well the respective average degree of polymerization (DPn). Fraction WM-F2.2 obtained with 53% of chloroform expressed a DPn of 8. Remain fractions were obtained with the increase of chloroform ratio. For 73% and 84% of chloroform, fraction WM-F2.4 and WM-F2.6 were obtained with DPn of 7 and 5, respectively. Fraction WA-F2.2, achieved with 58% of chloroform present a DPn of 7.

Table 4 – Grape seed phenolic fraction characterization: yield and DPn

Fraction	CHCl3 (%)	Yield (%)	Total Procyanidin (%, w/w)	Catechin (%)		Epicatechin (%)		Epicatechin-*O*-Gallate(%)		DPn
				Terminal Unit	Extension Units	Terminal Unit	Extension Units	Terminal Unit	Extension Units	
WM-F2.2	52	10.5	46.2	57.0	5.3	29.4	75.9	13.6	18.8	8
WM-F2.4	73	6.6	68.5	51.1	4.3	30.8	76.3	18.2	19.3	7
WM-F2.6	84	3.7	43.6	55.4	9.7	32.8	78.2	11.9	12.1	5
WA-F2.2	58	7.2	31.7	58.4	3.7	24.7	75.3	16.9	21.0	7

Fractions WM-F2.2 and WA-F2.2 had the same fractionation process with different extraction solvents, methanol and mixture acetone/water, respectively. As previously mentioned, methanol extraction would supposedly extract polyphenolic compounds of low molecular weight, while mixture of acetone/water, more polar, polymeric procyanidins. However, when analyzing DPn of each fraction, a higher DPn is found in methanol fraction. Pevic et al [29] studied the influence of the mixture of water to a solvent in grape seeds extraction. Pevic described that mixture of this solvent with water (co-solvent) increases significatively the extraction yield, due to the increase of permeability in the grape seed. The seed saturated with water allows a higher penetration of the solvent increasing consequently the extraction yield. Therefore, probably acetone/mixture isn't extracting longer

procyanidins but extracting, from grape seed matrix, inaccessible compounds by methanol, since the water increases permeability in grape seeds.

4.2. Angiotensin-I Converting Enzyme activity

4.2.1. Kinetic parameters

4.2.1.1. Km determination

To determine K_m of this reaction a range of substrate concentrations was prepared and the velocity of the reaction with these concentrations was plotted. Figure 20 shows initial velocities in relation to the range of substrate concentrations.

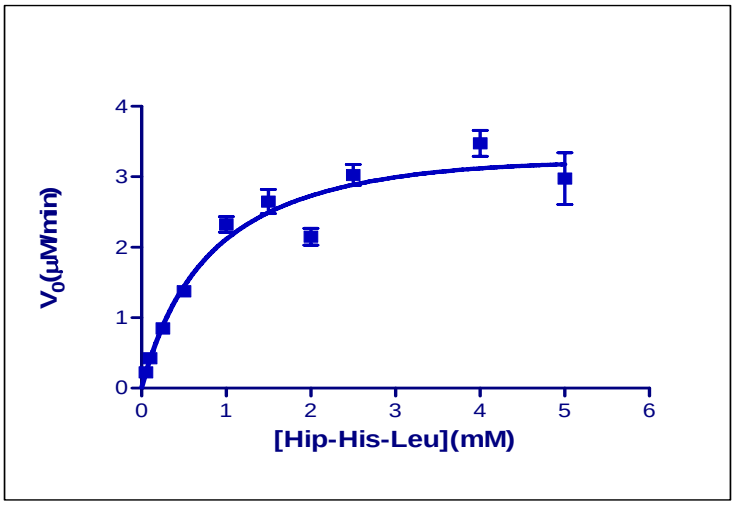


Figure 20 – Initial velocity in relation to substrate concentration

The determination of Km can be achieved by analytic representation methods , as Lineweaver-Bulk, Hanes Method or Eadie-Scatchard Method, which use graphic representation to determined $V_{\max}$ and K_m. In this work, determination of $V_{\max}$ and K_m was obtained adjusting results to one-site binding (hyperbole) and results were given by GraphPad 5.0 routines. A K_m of $0.77mM$ and a $V_{\max}$ of $3.68\mu M.\min^{-1}$ was obtained.

4.2.1.2. Time-course

A time-course curve permits to establish the optimum time reaction. A time reaction of 30 minutes was decribed by Tsutsumi [35]. A curve representing His-Leu formation in relation to time is given in Figure 21. This figure shows that till 30 minutes of substrate-enzyme incubation the formation of His-Leu highly increases (almost 85% of total product formation) but after the 30 minutes, the

product formation becomes slower, with a slight increase till 45 min and after almost keeps constant. These results lead to maintenance of 30 minutes reaction period

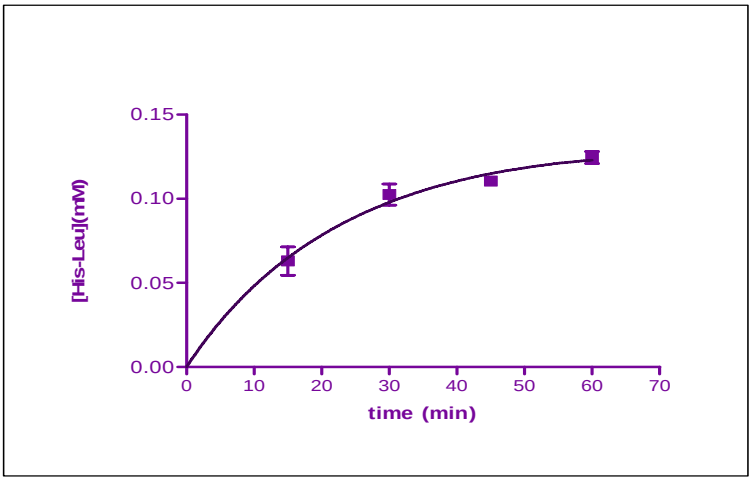


Figure 21 – His-Leu concentration (product formation) in relation to reaction time

4.2.2 Inhibition of purified ACE by captopril and pure monomeric phenolic compounds

After setting experimental conditions ACE activity was determined in the presence and the absence of inhibitors.

The inhibition of ACE as function of the captopril concentration is shown in Figure 22. Captopril showed a strong capacity to inhibit ACE. With only 5 nM of captopril, an inhibition of 32.2% is achieved. These results were not surprising since captopril is a synthetic drug commonly use in the treatment of hypertension.

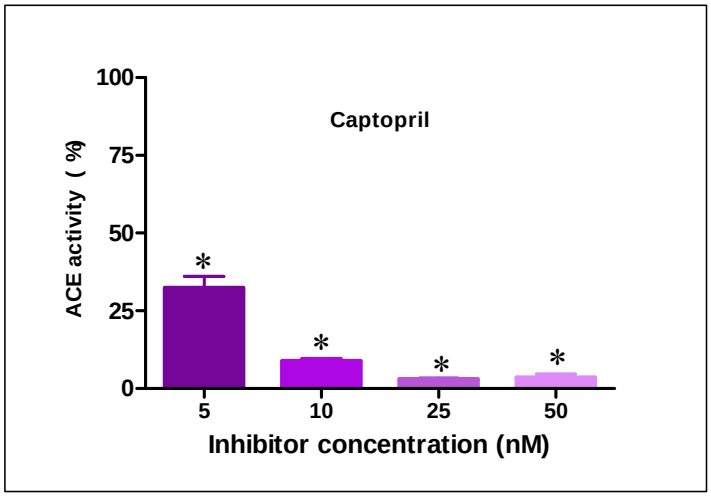
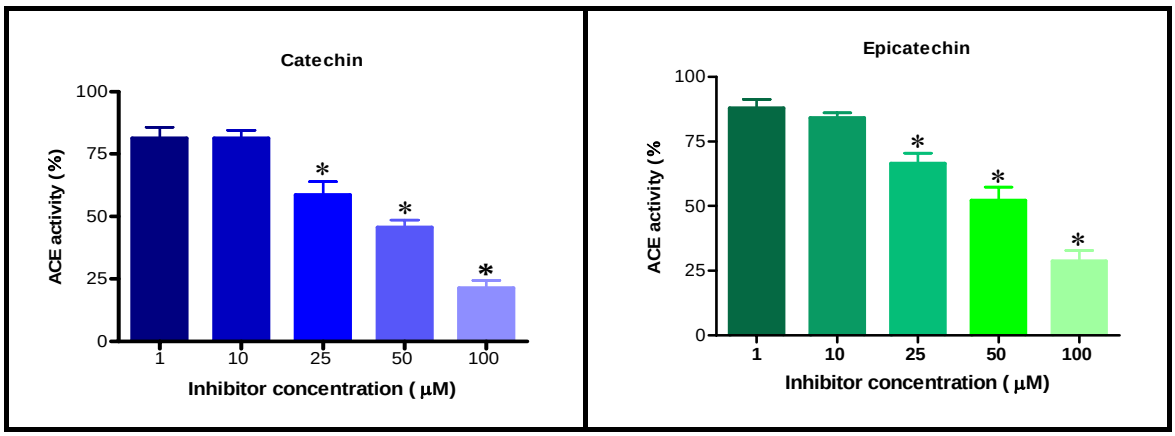


Figure 22 – Effect of the concentration of captopril on ACE activity. Columns and vertical lines represent mean ± SEM. Significantly different from control, * $p<0.05$.

In Figure 23, the results achieved with (+)-catechin, (-)-epicatechin, (+)-catechin-*O*-gallate, and (-)-epigallocatechin are plotted. The ACE activity for 100 μM of pure monomers was of 21.5%, 29.4%, 18.6% and 35.6%, for (+)-catechin, (-)-epicatechin, (+)-catechin-*O*-gallate and (-)-epigallocatechin, respectively.

The captopril effect is ca 1000-fold higher than that of the purified monomers studied, as it acts in the nanomolar range with $I = 67.7 - 97.0\%$, in contrast with the micromolar concentrations of the monomers for which $I = 5.5 - 81.4\%$.



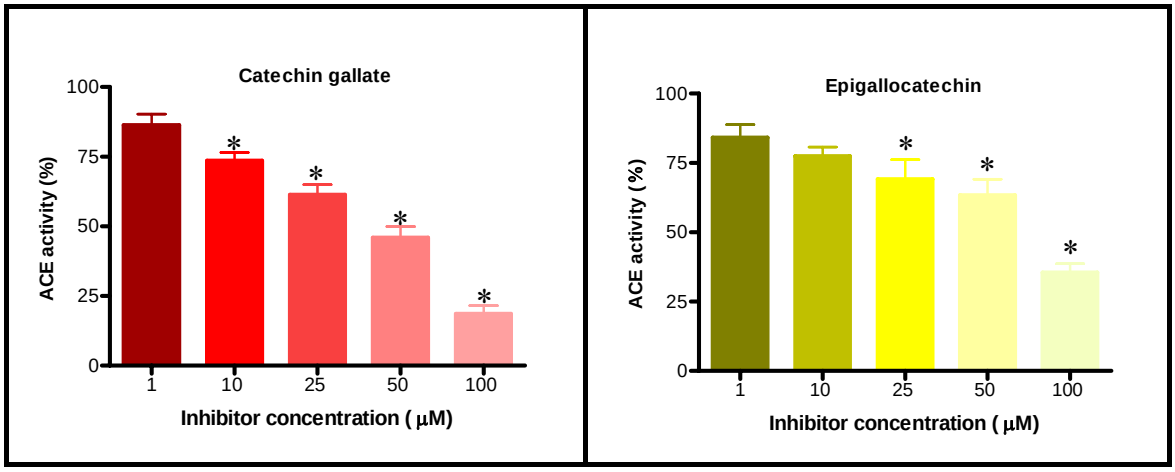


Figure 23 – Effect of the concentration of purified flavanols on ACE activity.

Regarding (+)-catechin, (+)-catechin-*O*-gallate, and (-)-epicatechin, Figure 24 shows that no statistically significant inhibition differences were found ($IC_{50} = 40.0$, 35.0, and 44.9 μM, respectively), whereas (-)-epigallocatechin presented the worst result, $IC_{50} = 59.3\ \mu M$.

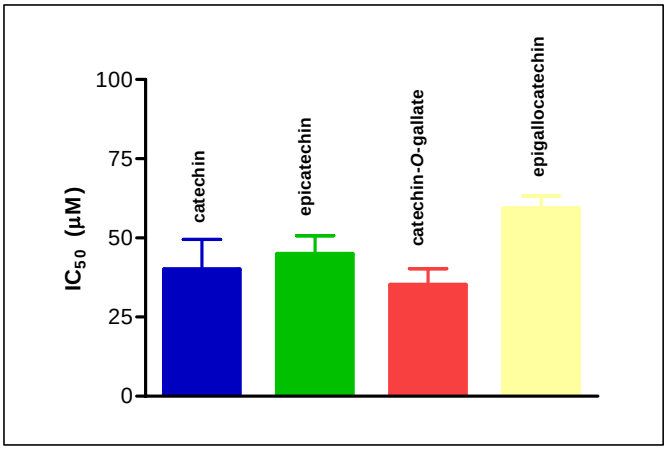


Figure 24 – Inhibitory Concentration 50 (IC50) calculated for the inhibition of ACE activity by purified flavanols. Columns and vertical lines represent mean ± SEM.

Although captopril, a synthetic compound, shows an undoubtedly greater capacity to inhibit ACE activity, the purified (+)-catechin, (-)-epicatechin, (+)-catechin-*O*-gallate, and (-)-epigallocatechin should also be taken into account, as 100 μM solutions decreased ACE activity to 18.6-35.6%. Furthermore, it is worth noting that these flavanols are natural compounds present in a wide variety of foods, which provides a source of ACE inhibitors without adverse side effects. For instance, captopril is commonly associated to cough (the most common side effect), rash and taste disturbances (metallic or loss of taste), which are attributed to the unique sulfhydryl moiety [58].

4.2.3 Inhibition of purified ACE by grape seed extracts

In Figure 25 the inhibition of ACE against the solution concentration of each natural extract is illustrated in individual graphics; their calculated IC_{50} are plotted in Figure 26. It is possible to observe that WM-F2.2 shows the strongest effect since $IC_{50} = 0.12$ mg/L, while WM-F2.4, WA-F2.2, and WM-F2.6 present $IC_{50} = 0.39$, 2.26 and 2.65 mg/L, respectively. The inhibition measured at 3.0 mg/L for the same fractions were 98.1, 63.4, 56.4, and 52.7%, respectively; at 30.0 mg/L, $I \cong 100\%$ for WM-F2.2, WM-F2.4, WM-F2.6 and $I = 94.3\%$ for WA-F2.2.

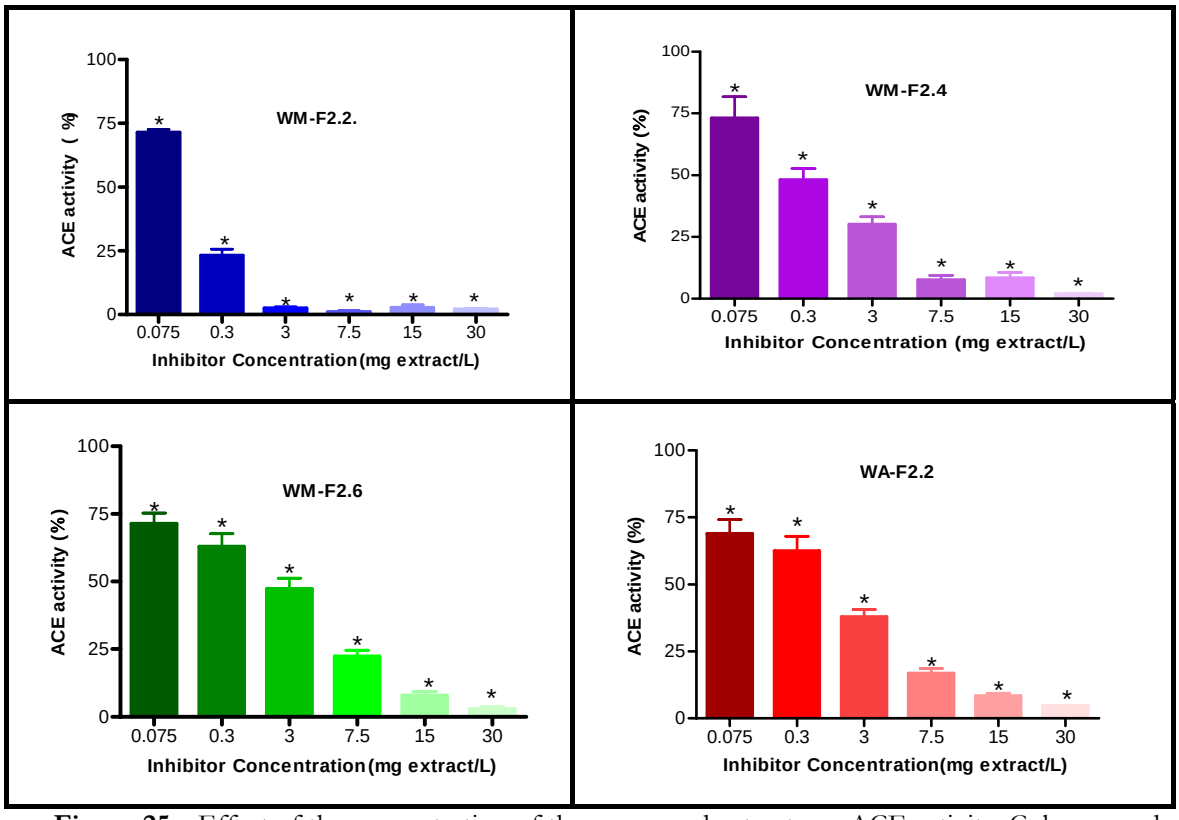


Figure 25 – Effect of the concentration of the grape seed extracts on ACE activity. Columns and vertical lines represent mean ± SEM. Significantly different from control, * $p<0.05$.

The fractions of grape seed extracts studied in this work were previously characterized by HPLC-UV[50]. These procyanidins are essentially constituted by flavan-3-ols units of (+)-catechin, (-)-epicatechin, and (-)-epicatechin-*O*-gallate. The fractions tested possess different average degrees of

polymerization (DPn), namely: DPn(WM-F2.2) = 8, DPn(WM-F2.4) = 7, DPn(WM-F2.6) = 5, and DPn(WA-F2.2) = 7.

Studies comparing the inhibitory capacity of monomers and procyanidins refer the notion that higher molecular weight compounds are more effective, as well as the idea the inhibition extension could be associated with the number of hydroxyl groups available to establish hydrogen bonds with ACE protein [33]. Our results corroborate such finding, as the grape seed extracts studied exhibited stronger capacity than the pure monomers tested. In fact, attending to Figures 24 and 26, the IC_{50} of (+)-catechin, (-)-epicatechin, (+)-catechin-*O*-gallate, and (-)-epigallocatechin are 40.0, 44.9, 35.0, 59.3 μM, which in units of mg/L correspond to 12.0, 13.5, 10.5, and 17.8, whereas for WM-F2.2, WM-F2.4, WM-F2.6, and WA-F2.2 the IC_{50} are 0.12, 0.39, 2.65 and 2.26 mg extract/L, respectively. Furthermore, results from Figures 23 and 25 show that, for the same concentrations, some natural phenolic extracts promote almost total inhibition, whereas flavanols do not. For instance, at 3.0 mg/L, $I(\text{WM - F2.2}) = 98.1\%$ whereas pure monomers reached only 14.6-25.5%; at 30.0 mg/L, $I(\text{WM - F2.6}) = 97.8\%$ and $I(\text{WA - F2.2}) = 94.3\%$, against 68.0-84.0% measured for the phenolic monomers.

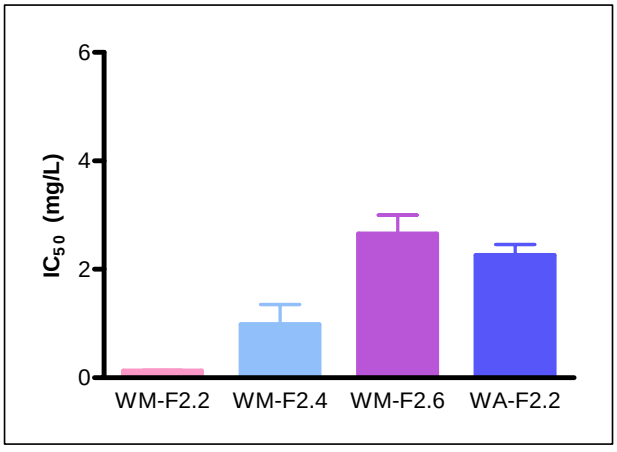


Figure 26 – Inhibitory Concentration 50 (IC_{50}) calculated for the inhibition of ACE activity by white grape seed extracts. Columns and vertical lines represent mean ± SEM.

The IC_{50} and DPn of the procyanidins extracts are simultaneously represented in Figure 27. Results evidenced that there exists a connection between both variables, as IC_{50} increases with increasing DPn. Larger molecules, i.e. polyphenolics with higher average degree of polymerization, may be associated to greater inhibitory effect: for WM-F2.6, $DPn = 5$ and $IC_{50} = 0.12 mg/L$; for

WM-F2.4 and WA-F2.2, $DPn = 7$, and $IC_{50} = 0.39 mg/L$ and 2.26%, respectively; finally, WM-F2.2 presents DPn = 8 and $IC_{50} = 2.65 mg/L$. The influence of the number of monomeric units in the extension and specificity of ACE inhibition has been already suggested by Actis-Goretta et al.[33].

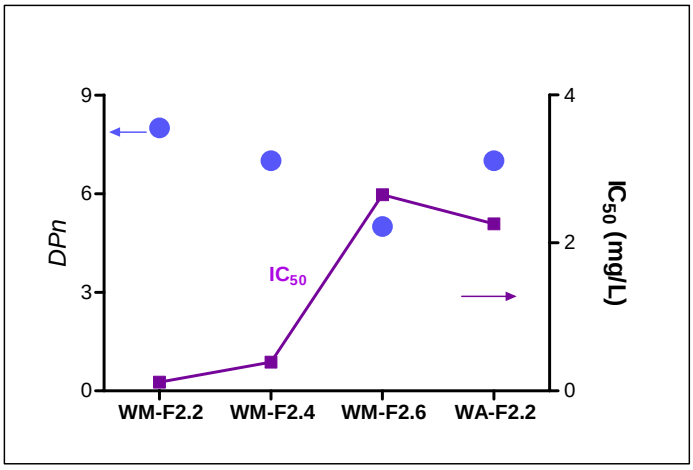


Figure 27 – Inhibitory Concentration 50 (IC_{50}) calculated for the inhibition of ACE activity by the white grape seed extracts WM-F2.2, WM-F2.4, WM-F2.6, and WA-F2.2, represented along with the average degree of polymerization of each extract.

Larger procyanidins seem to exert broader interactions on protein surfaces. A larger procyanidin reach certain areas of the enzyme that cannot be accessed by a monomer. In that study, the authors suggest the more (-)-epicatechin units the procyanidin structure provides, further active groups interact with the enzyme, increasing both the affinity for the enzyme and the number of binding sites that can be recognized on the ACE surface.

In this essay, results evidenced that the number of polymerized units, and consequently the absolute number of heterocyclic oxygen and hydroxyl groups present in solution, can not be the unique explanation for the effective behavior of our natural extracts, since their concentrations are clearly lower than those of pure monomers: e.g., $IC_{50}(\text{natural extracts}) = 0.12–2.65$ mg/L, against $IC_{50}(\text{pure monomers}) = 35.0–59.3$ mg/L. In the following, another two factors possibly involved in the inhibition mechanism will be discussed.

Studies evaluating the inhibitory action of procyanidins in the presence of albumin (since albumin has the ability to bind procyanidins) revealed that small oligomeric compounds (dimers and tetramers) were not affected in their inhibitory capability over ACE, while the potential of hexamers was highly reduced[34]. Such results emphasize the important role played by the size of these molecules. Concerning the mechanism involved, Wei et al.[59] reported the efficacy of ACE inhibitors may vary depending upon the competition with the substrate for the N- or C-terminal active sites of

ACE. A kinetic analysis suggested that flavan-3-ols and procyanidins do compete with substrate for the active sites of ACE, indicating that larger molecules can inhibit both C- and N-active sites to a similar extent, while monomers seem to inhibit preferentially N-active sites [33].

The number of hydroxyl groups in the procyanidins may be also related with their interaction with the surface of the enzyme, since the hydroxyl group determines the molecules capability to adsorb onto the cell membranes [6,33]. The possible combinations of putative zinc-chelating functional groups should be considered between the several flavan-3-ol units. Tsutsumi et al. [35] suggested that the phenolic OH- at the C7 position on the aromatic A ring, and the heterocyclic oxygen atom are putative zinc-chelating sites – see structural formula of a procyanidin dimer in Figure 5. These authors found the common characteristic of seven phenolic compounds screened seemed to be these groups. Therefore, larger molecules provide more hydroxyl and heterocyclic oxygen groups for the interactions described.

The greater effect evidenced by the grape seed extracts could be also explained by a synergetic effect of the various molecules present in each fraction. An analogous behaviour on the activity of ACE was referred by Persson et al. [60], who studied the combination of low non-effective concentrations of ginseng (0.1 mg/mL) with enalaprilat (10^{-11} M). The results showed a significant reduction of the ACE activity in human cultured endothelial cells from umbilical cords (HUVEC) in relation to that measured with enalaprilat alone, specifically from 5.5 to nearly 2 $\mu M / \min$. It is worth noting that the procyanidins extracts were characterized here by the average degree of polymerization, which allowed us to draw some conclusions that corroborate other studies reported in the literature. However the number of monomers does not define uniquely the specificity of ACE inhibition. The way how the monomeric units are bound should also be considered, as Ottaviani et al. [34] showed that (-)-epicatechin dimers with different linkage bound structure had completely different effects in the activity of ACE.

Chapter 5 – Conclusion

The aim of this work was to analyse two components of grape seed: oil and polyphenolic compounds. The potential value of this two components assures that this by-product of wine industry, is treated not as a residue but as a valuable product for others applications (as health and nutrition).

The two components were extracted and characterized. Grape seed, *Vitis vinifera* L, Chardonnay, white variety, presented 11.6% of oil and 25.5% of a extract rich in polyphenolic compounds.

Oil characterization showed that grape seed oil present 77.2% of polyunsaturated acids, in the form of triglycerides. A high concentration of unsaturated fatty acids makes grape seed oil an edible oil with good characteristics for nutritional use. In fact, grape seed oil present 60% of essential fatty acids, with 59.8% of linoleic acid and 0.5% of. linolenic acid and the monounsaturated fatty acid, oleic acid (17.4%).

Polyphenolic compounds from grape seed present flavan-3-*O*-ol, in the form of procyanidins (polymerized forms of (+)-catechin, (-)-epicatechin and (-)-epicatechin-3-*O*-gallate). Characterization of polyphenolic permitted, not only define the constituents, but also obtain fractions with different average degrees of polymerization. The presence of procyanidins in the polyphenolic extract led to the study of the inhibitory capacity of this procyanidins in Angiotensin I-Converting Enzyme.

The effect of polyphenolic compounds on the activity of the angiotensin I-converting enzyme was studied, namely four purified monomers ((+)-catechin, (-)-epicatechin, (+)-catechin-*O*-gallate, and (-)-epigallocatechin) and four procyanidin extracts of grape seeds (WM-F2.2, WM-F2.4, WM-F2.6, and WA-F2.2). The natural fractions produced a strong inhibitory effect upon ACE, which was interestingly stronger than that measured with the pure monomers. The IC_{50} of the monomers are in the range 35.0-59.3 mg/L (or 40.0-59.3 μM), whereas the procyanidin values can be 500 times lower (between 0.12 and 2.65 mg/L). Another important result was the connection found between the inhibitory effect of our natural extracts and their average degree of polymerization: the inhibition of ACE activity increases with increasing DPn. A possible synergetic effect and the increasing number of units in the oligomeric compounds could contribute in part to such results, since the heterocyclic oxygen and the hydroxyl groups present in their structural units may be the binding sites recognized on the ACE surface. Hence, in the DPn range of water soluble procyanidins, larger molecules give rise to higher inhibition.

References

[1] Corma, A., Iborra, S. and Velty, A. (2007). Chemical routes for the transformation of Biomass into Chemicals. Chemical Reviews 107, 2411-2502.

[2] Huang, H.J., Ramaswamy, S., Tschirner, U.W. and Ramarao, B.V. (2008). A review of separation technologies in current and future biorefineries. Separation and Purification Technology 62, 1-21.

[3] Gabinete de Planeamento e Políticas (2007) Vitivinicultura - Diagnóstico Sectorial - Ministério da Agricultura e do Desenvolvimento Rural e Pescas.

[4] Palma, M., Taylor, L.T., Varela, R.M., Cutler, S.J. and Cutler, H.G. (1999). Fractional extraction of compounds from grape seeds by supercritical fluid extraction and analysis for antimicrobial and agrochemical activities. J Agric Food Chem 47, 5044-8.

[5] Murga, R., Ruiz, R., Beltran, S. and Cabezas, J.L. (2000). Extraction of natural complex phenols and tannins from grape seeds by using supercritical mixtures of carbon dioxide and alcohol. J Agric Food Chem 48, 3408-12.

[6] Actis-Goretta, L., Ottaviani, J.I. and Fraga, C.G. (2006). Inhibition of angiotensin converting enzyme activity by flavanol-rich foods. Journal of Agricultural and Food Chemistry 54, 229-234.

[7] Wong, J., Patel, R.A. and Kowey, P.R. (2004). The clinical use of angiotensin-converting enzyme inhibitors. Prog Cardiovasc Dis 47, 116-30.

[8] Matthaus, B. (2008). Virgin grape seed oil: Is it really a nutritional highlight. European Journal of Lipid Science Technology 110, 645-650.

[9] Metzger, J.O. and Bornscheuer, U. (2006). Lipids as renewable resources: current state of chemical and biotechnological conversion and diversification. Appl Microbiol Biotechnol 71, 13-22.

[10] Horrobin, D.F. and Manku, M.S. (1983). How do polyunsaturated fatty acids lower plasma cholesterol levels? Lipids 18, 558-62.

[11] Crews, C., Hough, P., Godward, J., Brereton, P., Lees, M., Guiet, S. and Winkelmann, W. (2006). Quantitation of the main constituents of some authentic grape-seed oils of different origin. J Agric Food Chem 54, 6261-5.

[12] Baydar, N., Ozkan, O.,Çetin, E. . (2007). Characterization of grape seed and pomace oil extracts. Grasas Y Aceites 58, 29-33.

[13] Luque-Rodriguez, J., Luque de Castro, M., Pérez-Juan, P. (2005). Extraction of fatty acids from grape seed by superheated hexane. Talanta 68, 126-130.

[14] Wang, L., Weller, C. (2006). Recent advances in extraction of nutraceuticals from plants. Trends in Food Science & Technology 17, 300-312.

[15] Mamidipally, P., Liu, S. (2004). First approach on rice bran oil extraction using limonene. European Journal of Lipid Science Technology 106, 122-125.

[16] Hanmoungjai, P., Pyle, L., Niranjan, K. (2000). Extraction of rice bran oil using aqueous media. Journal of Chemical Technology and Biotechnology 75, 348-352.

[17] Luque-Garcia, J., Luque de Castro, M. (2004). Ultrasound-assisted Soxhlet extraction: An expeditive approach for solid sample treament - Application to the extraction of total fat from oleaginous seeds. Journal of Chromatography A 1034, 237-242.

[18] Gomez, R.A., Chevalier, R.L., Carey, R.M. and Peach, M.J. (1990). Molecular biology of the renal renin-angiotensin system. Kidney Int Suppl 30, S18-23.

[19] Schuchardt, U., Sercheli, R., Vargas, R. (1998). Transesterification of Vegetables Oils: a Review. J. Braz. Chem. Soc. 9, 199-210.

[20] Geleijnse, J.M., Launer, L.J., Hofman, A., Pols, H.A. and Witteman, J.C. (1999). Tea flavonoids may protect against atherosclerosis: the Rotterdam Study. Arch Intern Med 159, 2170-4.

[21] Hertog, M.G., Feskens, E.J., Hollman, P.C., Katan, M.B. and Kromhout, D. (1993). Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study. Lancet 342, 1007-11.

[22] Liu, Z., Ma, L.P., Zhou, B., Yang, L. and Liu, Z.L. (2000). Antioxidative effects of green tea polyphenols on free radical initiated and photosensitized peroxidation of human low density lipoprotein. Chem Phys Lipids 106, 53-63.

[23] Arts, I.C., Hollman, P.C., Bueno De Mesquita, H.B., Feskens, E.J. and Kromhout, D. (2001). Dietary catechins and epithelial cancer incidence: the Zutphen elderly study. Int J Cancer 92, 298-302.

[24] Cirico, T.L. and Omaye, S.T. (2006). Additive or synergetic effects of phenolic compounds on human low density lipoprotein oxidation. Food Chem Toxicol 44, 510-6.

[25] Montealegre, R., Peces, R, Vozmediano, J, Gascuena, Romero, E. (2006). Phenolic compounds in skins and seeds of ten grape Vitis vinifera varieties grown in a warm climate. Journal of Food Composition and Analysis 19, 687-693.

[26] Fuleki, T., Ricardo da Silva, J. (1997). Catechin and Procyanidin composition of seeds from grape cultivars grown in Ontario. J Agric Food Chem 45, 1156-1160.

[27] Prieur, C., Rigaud, J., Cheynier, V., Moutounet, M. (1994). Oligomeric and polymeric procyanidins from grape seeds. Phytochemistry 36, 781-784.

[28] Escribano-Bailon, T., Santos-Buelga, C. (2003). Methods in polyphenol analysis. Polyphenol extraction from foods. Royal Society of Chemistry, 1-15.

[29] Pekic, B., Kovac, V., Alonso, E. and Revilla, E. (1998). Study of the extraction of proanthocyanidins from grape seeds. Food Chemistry 61, 201-206.

[30] Li, H., Pordesimo, L., Weiss, J. (2004). High intensity ultrasound-assisted extraction of oil from soybeans. Food Research International 37, 731-738.

[31] Haslam, E. (1974). Polyphenol-protein interactions. Biochem J 139, 285-288.

[32] Thompson, R., Jacques, D., Haslam, E., Tanner, D. (1972). Plant proanthocyanidins. Part I. Introduction; the isolation, structure and distribution in nature of plant procyanidins. J Chem Soc 1378.

[33] Actis-Goretta, L., Ottaviani, J.I., Keen, C.L. and Fraga, C.G. (2003). Inhibition of angiotensin converting enzyme (ACE) activity by flavan-3-ols and procyanidins. FEBS Lett 555, 597-600.

[34] Ottaviani, J.I., Actis-Goretta, L., Villordo, J.J. and Fraga, C.G. (2006). Procyanidin structure defines the extent and specificity of angiotensin I converting enzyme inhibition. Biochimie 88, 359-365.

[35] Tsutsumi, Y., Shimada, A., Miyano, A., Nshida, T., Mitsunsga, T. (1998). In vitro screening of angiotensin I-converting enzyme inhibitors from Japanese cedar (Cryptometria japonica). Journal of Wood Science 44, 463-468.

[36] Nijveldt, R.J., van Nood, E., van Hoorn, D.E., Boelens, P.G., van Norren, K. and van Leeuwen, P.A. (2001). Flavonoids: a review of probable mechanisms of action and potential applications. Am J Clin Nutr 74, 418-25.

[37] Dive, V. et al. (1999). RXP 407, a phosphinic peptide, is a potent inhibitor of angiotensin I converting enzyme able to differentiate between its two active sites. Proc Natl Acad Sci U S A 96, 4330-5.

[38] Corvol, P., Jeunemaitre, X., Charru, A., Kotelevtsev, Y. and Soubrier, F. (1995). Role of the renin-angiotensin system in blood pressure regulation and in human hypertension: new insights from molecular genetics. Recent Prog Horm Res 50, 287-308.

[39] Danilov, S., Jaspard, E., Churakova, T., Towbin, H., Savoie, F., Wei, L. and Alhenc-Gelas, F. (1994). Structure-function analysis of angiotensin I-converting enzyme using monoclonal antibodies. Selective inhibition of the amino-terminal active site. J Biol Chem 269, 26806-14.

[40] Ardaillou, R. and Michel, J.B. (1999). The relative roles of circulating and tissue renin-angiotensin systems. Nephrol Dial Transplant 14, 283-6.

[41] Nishimura, H. (2001). Angiotensin receptors--evolutionary overview and perspectives. Comp Biochem Physiol A Mol Integr Physiol 128, 11-30.

[42] Barbosa-Filho, J., Martins, V., Rabelo, L., Moura, M., Silva, M., Cunha, E., Souza, M., Almeida, R., Medeiros, I. (2006). Natural products inhibitorsof angiotensin converting enzyme(ACE). A review between 1980-2000. Brazilian Journal of Pharmacognosy 16, 421-446.

[43] De Gasparo, M., Catt, K., Inagami, T., Wright, J., Unger, T. (2000). The angiotensin II receptors. Pharmacol Rev 52, 415-472.

[44] Moreau, M.E., Garbacki, N., Molinaro, G., Brown, N.J., Marceau, F. and Adam, A. (2005). The kallikrein-kinin system: current and future pharmacological targets. J Pharmacol Sci 99, 6-38.

[45] Soubrier, F., Wei, L., Hubert, C., Clauser, E., Alhenc-Gelas, F. and Corvol, P. (1993). Molecular biology of the angiotensin I converting enzyme: II. Structure-function. Gene polymorphism and clinical implications. J Hypertens 11, 599-604.

[46] Guimarães, S., Moura, D., Soares-da-Silva, P. (2006) Terapêutica Medicamentosa e suas Bases Farmacologicas(edition, t., ed.^eds). Porto Editora

[47] Cushman, D.W. and Cheung, H.S. (1971). Spectrophotometric assay and properties of the angiotensin-converting enzyme of rabbit lung. Biochem Pharmacol 20, 1637-48.

[48] Chiknas, S.G. (1979). A liquid chromatography-assisted assay for angiotensin-converting enzyme (peptidyl dipeptidase) in serum. Clin Chem 25, 1259-62.

[49] Friedland, J. and Silverstein, E. (1976). A sensitive fluorimetric assay for serum angiotensin-converting enzyme. Am J Clin Pathol 66, 416-24.

[50] Passos, C., Cardoso, S., Barros, A., Silva, C., Coimbra, M. (2008 submitted). Procyanidin Average Degree of Polymerization using FT-IR Spectroscopy and O-PLS/PLS1 Regression.

[51] Povh, N., Marques, M., Meireles, M. (2001). Supercritical CO2 extraction of essential oil and oleoresin from chamomile (Chamomilla recutita [L.]. The Journal of Supercritical Fluids 21, 245-256.

[52] Guyot, S., Doco, T., Souquet, JM., Moutounet, M., Drilleau, JF. (1997). Characterization of highly polymerized procyanidins in cider apple (malus silvestris var. kermerrien) skin and pulp. Phytochemistry 44, 351-357.

[53] Saucier, C., Mirabel, M., Daviaud, F., Longieras, A. and Glories, Y. (2001). Rapid fractionation of grape seed proanthocyanidins. J Agric Food Chem 49, 5732-5.

[54] Peng, Z., Hayasaka, Y., Iland, P.G., Sefton, M., Hoj, P. and Waters, E.J. (2001). Quantitative analysis of polymeric procyanidins (Tannins) from grape (Vitis vinifera) seeds by reverse phase high-performance liquid chromatography. J Agric Food Chem 49, 26-31.

[55] Fiori, L. (2007). Grape seed oil supercritical extraction kinetic and solubility data: critical approach and modeling. The journal of supercritical fluids 43, 43-54.

[56] Ohnishi, M., Hirose, S., Kawaguchi, Ito, S., Fujino, Y. (1990). Chemical Composition of Lipids, Especially Triacylglycerol, in Grape Seeds. Agric. Biol. Chem. 54

[57] Passos, C.P., Cardoso, S.M., Domingues, M.R.M., Domingues, P., Silva, C.M. and Coimbra, M.A. (2007). Evidence for galloylated type-A procyanidins in grape seeds. Food Chemistry 105, 1457-1467.

[58] Atkinson, A.B. and Robertson, J.I.S. (1979). Captopril in the Treatment of Clinical Hypertension and Cardiac-Failure. Lancet 2, 836-839.

[59] Wei, L., Clauser, E., Alhencgelas, F. and Corvol, P. (1992). The 2 Homologous Domains of Human Angiotensin-I-Converting Enzyme Interact Differently with Competitive Inhibitors. Journal of Biological Chemistry 267, 13398-13405.

[60] Persson, I., Dong, L., Persson, K. (2005). Effect of Panax ginseng extract (G115) on angiotensin I-converting enzyme (ACE) activity and nitric oxide (NO) production. Journal of Ethnopharmacology 105, 321-325.